

Special Issue Reprint

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# Phytochemistry and Pharmacological Properties of Medicinal Plants

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Edited by  
Alfred Maroyi

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# **Phytochemistry and Pharmacological Properties of Medicinal Plants**



# Phytochemistry and Pharmacological Properties of Medicinal Plants

Guest Editor

**Alfred Maroyi**



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*Guest Editor*

Alfred Maroyi

Department of Biotechnology

and Biological Sciences

University of Fort Hare

Alice

South Africa

*Editorial Office*

MDPI AG

Grosspeteranlage 5

4052 Basel, Switzerland

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# About the Editor

## **Alfred Maroyi**

Alfred Maroyi is a full-time professor in the Department of Biotechnology and Biological Sciences, University of Fort Hare, South Africa. He obtained his Ph.D. in Ethnobotany and Plant Systematics from Wageningen University, the Netherlands. In his research, he focuses on ethnobotanical uses of plants and the phytochemical and pharmacological properties of plant species. Throughout his career spanning over three decades, he has published more than 200 peer-reviewed papers, over 30 book chapters, and co-edited two books. He serves on the editorial boards of several international journals.



# Preface

This collection on “Phytochemistry and Pharmacological Properties of Medicinal Plants” focuses on contributions that explore the ethnobotanical uses of medicinal plants, their phytochemical profiling, biological activities of medicinal plant extracts and/or their phytochemical compounds, safety and efficacy studies, as well as their cultural and ecological context. The contributions included here provide an updated and comprehensive overview of the ethnopharmacological properties of medicinal plants. This Special Issue aims to offer readers an integrated understanding of the medicinal uses, active ingredients, and pharmacological effects of selected plant species used as traditional medicines, and thus highlights the innovative diagnostic and therapeutic strategies that are currently shaping ethnopharmacological research. The motivation behind the creation of this Special Issue was, and continues to be, bridging Indigenous or traditional healing practices with modern and advanced research aimed at drug discovery and development. In response to these challenges, several passionate scientists from Bosnia and Herzegovina, Dominican Republic, Germany, Greece, Iran, Italy, Lebanon, Madagascar, Malaysia, Pakistan, Philippines, Poland, Republic of Korea, Russia, Saudi Arabia, Serbia, Slovakia, South Africa, Spain, Taiwan, Thailand, Tunisia, United Arab Emirates and United States of America set out to compile and disseminate research that explores utilization of plants as herbal medicines, their phytochemical, pharmacological and toxicological properties. Consequently, following a careful and thorough peer-review process, this Special Issue offers 16 original papers or reviews on several aspects of medicinal plants, their phytochemistry, and pharmacological and toxicological properties. I hereby extend heartfelt gratitude to all the authors and reviewers who have made this work possible. This Special Issue is intended for a diverse interdisciplinary audience—academic researchers, industry professionals, policymakers, other scientists, students, and anyone with an interest in medicinal uses of plants, their active ingredients, and pharmacological effects.

**Alfred Maroyi**

*Guest Editor*



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*Editorial*

# Phytochemistry and Pharmacological Properties of Medicinal Plants

Alfred Maroyi

Department of Biotechnology and Biological Sciences, University of Fort Hare, Alice 5700, South Africa; amaroyi@ufh.ac.za

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Medicinal plants have a long history of usage in the treatment and management of human and animal diseases and ailments. About 80% of people in both developed and developing countries depend on traditional medicines for their primary health care needs [1]. The therapeutic value of medicinal plants and their effectiveness in the treatment and management of human and animal diseases and ailments have been recognized around the world among different traditional medicinal systems such as those of Africa, North Africa, the Middle East, China, India (such as Ayurvedic medicine), Europe, America, Australia and Southeast Asia [2]. Traditional medicines, plant-derived medicines, other natural products and their derivatives are becoming increasingly popular in modern society as natural alternatives to synthetic chemicals [2]. Increasing acceptance of traditional medicine, plant-derived medicines and other natural products is often attributed to their deep-rooted cultural significance, affordability, and accessibility to the larger population in both developed and developing countries. This heavy reliance of local communities on traditional medicine reflects both the historical development of the Indigenous or traditional pharmacopeia and complex and dynamic human–nature relationships. Similarly, in other countries like South Africa, the use of medicinal plants and their extracts are an important component of the traditional pharmacopeia or traditional *Materia medica* and are also an aspect of the daily lives of many local people and an essential part of the South African cultural heritage [3]. In this context, knowledge about the identity of plant species used as sources of traditional medicines is important, as well as their medicinal applications, the plant parts used, and the dosage and administration. Pharmaceutical research, therefore, has brought about major shifts in perceptions of traditional medicine and significant changes concerning how this natural resource is utilized. This means that medicinal plants which historically were available only as bark, bulbs, leaves, roots, fruits, stems, rhizomes, tubers, corms, flowers, seeds, gums, exudates and nectar are today herbal medicines that are subjected to thorough ethnopharmacological research and development. The quality of a herbal medicine is directly related to its active secondary metabolites, commonly known as its phytocomplex [4]. A phytocomplex is a mixture of active and inactive molecules extracted from medicinal plant species characterized by specific biological activities and designed to harness the synergistic effects of all the molecules rather than a single isolated molecule. The biological activities of a phytocomplex are, therefore, stronger than the sum activities of a single active molecule [4]. Previous research has shown that phytocomplexes are characterized by synergy, several and various mechanisms of action, better bioavailability, minor toxicity and better tolerance [5]. In herbal medicine and phytotherapy research, phytoequivalence is often used to verify that a phytocomplex from a medicinal plant matches that of a clinically validated product [6]. Phytoequivalence is the concept that two herbal extracts are equivalent if they share matching phytochemical compounds and



identical biological activities [7]. Chromatographic techniques such as high-performance thin-layer chromatography (HPTLC) are often used to establish similarities between herbal extract profiles including in vitro or in vivo studies for efficacy [7].

The therapeutic applications of medicinal plants are often linked to their varied phytochemical properties. Numerous pharmaceutical drugs that are currently utilized as therapeutic agents and form the cornerstone treatments in contemporary clinical practice have been derived from natural products, particularly medicinal plants. Therefore, natural products serve as crucial sources of bioactive compounds that are therapeutically important and essential in pharmaceutical drug discovery research [8,9]. Well-known examples of plant derived medicines, plant drugs, phytomedicines or phytopharmaceuticals include artemisinin (*Artemisia annua* L.), codeine and morphine (*Papaver somniferum* L.), colchicine (*Colchicum autumnale* L.), combretastatin (*Combretum caffrum* (Eckl. & Zeyh.) Kuntze), digoxin (*Digitalis lanata* Ehrh.), paclitaxel (*Taxus brevifolia* Nutt.), reserpine (*Rauvolfia serpentina* (L.) Benth. ex Kurz, silymarin (*Silybum marianum* (L.) Gaertn.), vinblastine and vincristine *Catharanthus roseus* (L.) G. Don [3]. These phytomedicines, natural products and their derivatives provide the basis for more than 25% of all pharmaceutical drugs in clinical use in the world [3]. Therefore, an important and essential step aimed at evaluating the therapeutic significance of herbal medicine decoctions, infusions, syrups, juices, tinctures, steam inhalations and steam baths, powder snuffs, enemas, poultices, gargles, ointments, lotions, nasals and eyedrops involves isolation and identification of the pharmacologically active constituents of such medicinal plants, and this research approach forms the foundation of modern pharmaceutical development. This innovative phytopharmaceutical research coupled with state-of-the-art technologies has validated many of the healing attributes for which individual medicinal plant species have long been used traditionally. In general, all phytomedicines must have some pharmacological activity in order to be of therapeutic benefit for a particular disease or ailment [10].

Typically, many plant-based traditional medicines and natural products are accompanied by evidence-based biological efficacies, but there is often inadequate information about their standardized quality, mode of action, toxicology, how they interact with western medicines, evidence-based safety and regulatory approvals of these plant-based therapies [1,8,10,11]. The increased interest, trade and use of plant-based therapies and natural products prompted the World Health Organization [12] to draft internationally recognized guidelines for the quality assessment of herbal products, wherein governments of different countries are encouraged to promote the use of herbal medicines that have been scientifically validated for quality, safety and efficacy. Hence the need for pharmacological validation of plant-based traditional medicines and natural products through in vitro and in vivo and clinical studies. Clinical studies are the most convincing scientific method of proving the safety and efficacy of plant-based therapies and natural products [2].

Collectively, the contributions in this Special Issue reflect the diversity and depth of current research in phytochemistry and the pharmacological properties of medicinal plants. This Special Issue delves into the medicinal uses, phytochemical characterization, and pharmacological and toxicological properties of medicinal plants. Therefore, this compilation stimulates further research into traditional systems of medicine and how and why the plant species are used as herbal medicines, exploring their active phytochemical compounds and pharmacological properties using various in vitro and in vivo models, safety studies and clinical validation.

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# Medicinal Uses of the Fabaceae Family in Zimbabwe: A Review

Alfred Maroyi

Department of Botany, University of Fort Hare, Private Bag X1314, Alice 5700, South Africa; amaroyi@ufh.ac.za

**Abstract:** The current study is aimed at providing a systematic review of the ethnomedicinal, phytochemical and pharmacological properties of Fabaceae species used as sources of traditional medicines in Zimbabwe. Fabaceae is one of the well-known plant families of ethnopharmacological importance. Of the approximately 665 species of the Fabaceae family occurring in Zimbabwe, about 101 are used for medicinal purposes. Many communities in the country, mainly in peri-urban, rural and marginalized areas with limited access to healthcare facilities, rely on traditional medicines as their primary healthcare. The study reviewed research studies undertaken on Zimbabwe's Fabaceae species during 1959 to 2022. Information was gathered from literature sourced from Google Scholar, Science Direct, Scopus, PubMed, books, dissertations, theses and scientific reports. This study showed that 101 species are traditionally used to manage human and animal diseases in Zimbabwe. The genera with the highest number of medicinal uses are *Indigofera*, *Senna*, *Albizia*, *Rhynchosia* and *Vachellia*. Species of these genera are used as traditional medicines against 134 medical conditions, mainly gastrointestinal conditions, female reproductive conditions, respiratory conditions and sexually transmitted infections. Shrubs (39.0%), trees (37.0%) and herbs (18.0%) are the primary sources of traditional medicines, while roots (80.2%), leaves (36.6%), bark (27.7%) and fruits (8.9%) are the most widely used plant parts. Many of Zimbabwe's Fabaceae species used as sources of traditional medicines have been assessed for their phytochemical and pharmacological properties, corroborating their medicinal uses. However, there is a need to unravel the therapeutic potential of the family through further ethnopharmacological research focusing on toxicological studies, in vitro and in vivo models, biochemical assays and pharmacokinetic studies.

**Keywords:** Fabaceae; ethnobotany; herbal medicine; Leguminosae; natural compounds; traditional knowledge; Zimbabwe

## 1. Introduction

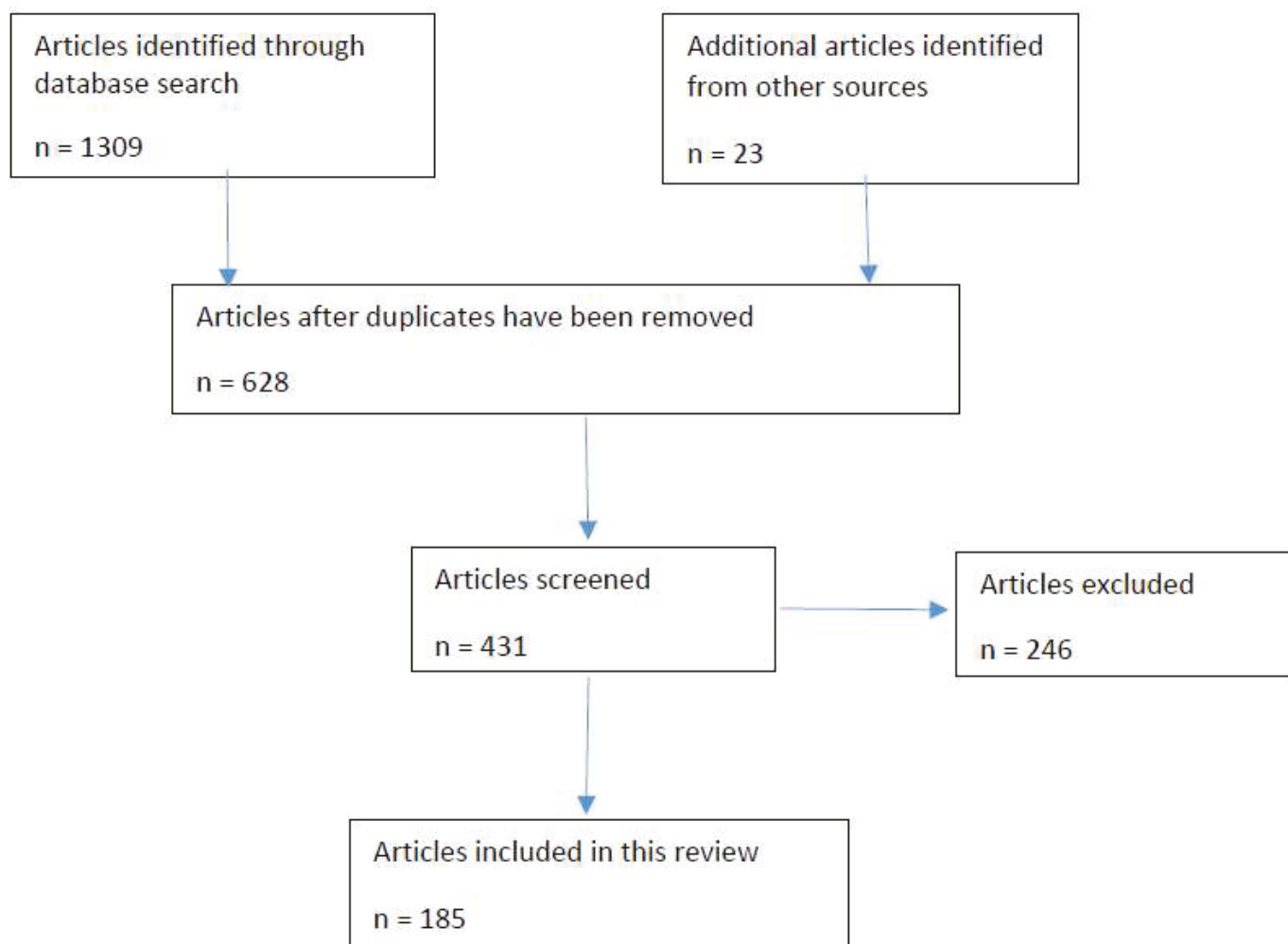
The Fabaceae (Leguminosae), often referred to as the bean, legume or pea family, is the third largest plant family after the Asteraceae and Orchidaceae in terms of plant species numbers [1]. The Fabaceae family consists of approximately 770 genera and 19,500 species [1,2] recorded in almost all of the biomes in the world except Antarctica and the high Arctic [3]. Research has shown that the success of the family in dominating in several hospitable and disturbed habitats is ascribed to the ability of the species to fix atmospheric nitrogen, thus allowing the plant species to grow in nutrient-poor soils [4–6]. Recent morphological and molecular research has supported that the Fabaceae family is a monophyletic family [2,7]. However, the Fabaceae family is divided into six subfamilies, namely the Caesalpinioideae (148 genera and 4400 species), Cercidoideae (12 genera and 335 species), Detarioideae (84 genera and 760 species), Dialioideae (17 genera and 85 species), Duparquetioideae (monotypic genus) and Faboideae (or Papilionoideae) (503 genera and 14,000 species) [2]. Members of the Fabaceae family include trees, shrubs, subshrubs, woody lianas, climbing annuals, herbs and aquatics [8]. The flowers are asymmetric, bilaterally symmetric or radially symmetric, and are pollinated by bats, birds and insects [9]. The leaves of the majority of species belonging to the Fabaceae family are compound, double-compound or trifoliolate, sometimes with a swollen leaf base, a superior ovary with one

locular, and the fruit is usually a two-valved, dehiscent pod that is rarely fleshy but is sometimes indehiscent and occasionally breaking into segments [10,11].

The majority of the members of the Fabaceae family are culturally and economically important throughout the world, and are used as sources of traditional medicines, food, timber, garden ornamentals, dyes, fibres, fuels, gums and insecticides [6,12,13]. The role played by Fabaceae species in the provision of ecosystem services and goods that support human wellbeing and survival have been highlighted in some studies conducted in different countries of the world [14,15]. Many members of this family have been widely studied for their bioactive chemical constituents such as phenolic acids, flavonoids, lectins, saponins, alkaloids and carotenoids [16]. Pharmacological studies have shown that some species exhibit potent anticancer, antioxidant, antimicrobial, anti-inflammatory, analgesic, antiulcer, antidiabetic, antirheumatic, cytotoxic and antiparasitic activities, among others [16–18]. Therefore, extensive phytochemical and pharmacological evaluations of some of the utilized Fabaceae species may lead to the discovery and development of novel pharmaceutical products, functional food ingredients and cosmetic products. Despite the discovery of several secondary metabolites in the Fabaceae, this family has attracted disproportionately little attention in the context of ethnopharmacological research. It is, therefore, within this context that this study was undertaken, with the aim of exploring and documenting the ethnomedicinal knowledge of Zimbabwe. Such a synthesis identified the gaps in knowledge on the therapeutic potential of the Fabaceae species and may also provide helpful information on ethnopharmacological research areas that require further research.

## 2. Materials and Methods

A literature search on Fabaceae species used as traditional medicines in Zimbabwe was conducted from September 2021 to November 2022. This information was retrieved from different online databases such as BioMed Central, Web of Science, Springerlink, Google Scholar, Scielo, PubMed, Science Direct, ACS Publications, Scopus and JSTOR. In addition, theses, dissertations, book chapters, books and scientific reports were retrieved from the libraries of the University of Fort Hare (UFH) in South Africa and the National Herbarium (SRGH) in Harare, Zimbabwe. Keywords and terminologies such as Zimbabwe, ethnobotany, ethnomedicine, ethnopharmacology, indigenous, medicine, phytomedicine, traditional medicine, Zimbabwean Fabaceae, Zimbabwean Leguminosae, medicinal Fabaceae, medicinal Leguminosae, Zimbabwean traditional medicine, Fabaceae and Leguminosae were used to search for relevant articles as shown in the PRISMA flow diagram (Figure 1). From each article, the following information was collected: the scientific names of the plant species, their growth form, plant part(s) used, methods of preparation and medicinal uses. The medicinal use categories were classified according to the Economic Botany Data Collection Standard [19]. The scientific names of the Fabaceae species from the original data sources were updated to the recently accepted names according to the Plants of the World Online website [20]. The Fabaceae subfamilies were updated following the classifications of the “Legume Phylogeny Working Group”, which presently recognizes six subfamilies: Caesalpinioideae, Cercidoideae, Detarioideae, Dialioideae, Duparquetioideae and Papilionoideae [2].



**Figure 1.** Flow diagram showing the identification and screening of the articles used in this review.

### 3. Results and Discussion

#### 3.1. Medicinal Plant Diversity

This study recorded 101 species traditionally used to manage and treat human and animal diseases in Zimbabwe (Table 1). Of these, 91 species are indigenous to Zimbabwe (90.1%), while nine species are exotic (8.9%), either naturalized as weeds or cultivated in home gardens and agricultural fields as ornamentals, fodder or food plants. The subfamilies Caesalpinoideae and Faboideae are dominant, with 54 species (53.5%) and 55 species (54.5%), respectively, and the remaining two species belonging to the Cercidoideae. Therefore, 101 species (15.2%) out of 665 species of the Fabaceae family known to occur in Zimbabwe [21] are used as sources of traditional medicines. A similar study by Van Wyk [6] showed that 338 species out of 1748 Fabaceae species (19.3%) are used as traditional medicines in southern Africa. Similar findings have been reported in Thailand, where 261 species out of 688 Fabaceae species are used as sources of traditional medicines [22]. Macêdo et al. [23] and Sutjaritjai et al. [24] argued that the prominence of Fabaceae taxa in traditional pharmacopoeia throughout the world is possibly associated with the wide distribution of the family, as the different growth forms of the species grow in many types of habitats and vegetation, and therefore are available in all seasons.

Table 1. Medicinal Fabaceae plants of Zimbabwe.

| Plant Taxa                                                                     | Habit   | Parts Used                       | Medicinal Uses                                                                                                                                                                                                                                                                            | Literature Records | References    |
|--------------------------------------------------------------------------------|---------|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|---------------|
| <i>Abrus precatorius</i> L. subsp. <i>africanus</i> Verdc.<br>+1               | Climber | Leaves, roots and seeds          | Bilharzia (schistosomiasis), sexually transmitted infections (STIs) and lucky charms                                                                                                                                                                                                      | 4                  | [25–28]       |
| <i>Aeschynomene mimosifolia</i> Vatke <sup>1</sup>                             | Shrub   | Roots                            | Chest pains and headache                                                                                                                                                                                                                                                                  | 1                  | [25]          |
| <i>Afzelia quanzensis</i> Welw. <sup>2</sup>                                   | Tree    | Bark, fruits and root bark       | Bloated stomach, blood pressure, depressed fontanelle, haemorrhoids and stomach problems, lucky charms and painful udders in cattle                                                                                                                                                       | 2                  | [25,28]       |
| <i>Albizia amara</i> (Roxb.) Boivin <sup>2</sup>                               | Tree    | Bark, leaves and roots           | Aphrodisiac, constipation, diarrhoea, dilating the birth canal, dysentery, oedema, painful placenta, palpitations, pneumonia, purgative, stomach problems, tuberculosis (TB), warts, and protection against witchcraft                                                                    | 5                  | [25,28–31]    |
| <i>Albizia adianthifolia</i> (Schumach.) W.Wight <sup>2</sup>                  | Tree    | Roots                            | Ethnoveterinary medicine                                                                                                                                                                                                                                                                  | 2                  | [32,33]       |
| <i>Albizia anthelmintica</i> (A.Rich.) Brongn. <sup>2</sup>                    | Shrub   | Bark                             | Wounds                                                                                                                                                                                                                                                                                    | 1                  | [25]          |
| <i>Albizia antunesiana</i> Harms <sup>2</sup>                                  | Tree    | Bark, bark sap, leaves and roots | Abdominal pains, aphrodisiac, bilharzia, constipation, depressed fontanelle, diarrhoea, gonorrhoea, infertility in women, menstrual problems, painful legs, painful uterus, preventing abortion, purgative, sexually transmitted diseases (STDs), sore eyes, sore throat and swollen legs | 8                  | [25–28,34–37] |
| <i>Albizia tanganyicensis</i> Baker <sup>2</sup>                               | Tree    | Bark and roots                   | Cough and swollen legs                                                                                                                                                                                                                                                                    | 1                  | [25]          |
| <i>Albizia versicolor</i> Welw. ex Oliv. <sup>2</sup>                          | Tree    | Roots                            | Erectile dysfunction, infertility in men and sexual impotence                                                                                                                                                                                                                             | 3                  | [25,28,29]    |
| * <i>Arachis hypogaea</i> L. <sup>1</sup>                                      | Herb    | Leaves                           | Cataracts, infertility in women and sore eyes                                                                                                                                                                                                                                             | 1                  | [25]          |
| <i>Baikiaea plurijuga</i> Harms <sup>2</sup>                                   | Tree    | Bark                             | Bloated stomach and haemorrhoids                                                                                                                                                                                                                                                          | 1                  | [28]          |
| <i>Bauhinia galpinii</i> N.E.Br. <sup>3</sup>                                  | Shrub   | Roots and seeds                  | Infertility and menstrual problems                                                                                                                                                                                                                                                        | 2                  | [25,28]       |
| <i>Bauhinia petersiana</i> Bolle <sup>3</sup>                                  | Tree    | Roots                            | Depressed fontanelle, infertility in women, menstrual problems and preventing witchcraft                                                                                                                                                                                                  | 2                  | [25,28]       |
| <i>Bobgunnia madagascariensis</i> (Desv.) J.H. Kirkbr. & Wiersema <sup>1</sup> | Tree    | Fruits, pods and roots           | Abdominal pains, convulsions, diarrhoea, emetic, earache, headache, infertility in men and women, oedema, stomach problems, syphilis and wounds                                                                                                                                           | 3                  | [25,28,34]    |
| <i>Bolusanthus speciosus</i> (Bolas) Harms <sup>1</sup>                        | Tree    | Leaves                           | Bile emesis and emetic                                                                                                                                                                                                                                                                    | 1                  | [25]          |



Table 1. Cont.

| Plant Taxa                                                             | Habit | Parts Used                    | Medicinal Uses                                                                                                                                                                                                                                                                                                                            | Literature Records | References                |
|------------------------------------------------------------------------|-------|-------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|---------------------------|
| <i>Brachystegia boehmii</i> Taub. <sup>2</sup>                         | Tree  | Bark, leaves, roots and twigs | Abdominal pains, antivenom, back pain, cataracts, heart problems, mental problems, sore eyes, STIs, toothache, constipation and lumbago in ruminants                                                                                                                                                                                      | 5                  | [25,28,34,36,37]          |
| <i>Brachystegia spiciformis</i> Benth. <sup>2</sup>                    | Tree  | Bark, fibre and roots         | Constipation, diarrhoea, mental problems, pain, sore eyes and wounds                                                                                                                                                                                                                                                                      | 3                  | [25,28,38]                |
| <i>Burkea africana</i> Hook. <sup>2</sup>                              | Tree  | Bark, leaves and roots        | Abdominal pains, anti-inflammatory, bilharzia, cancer, diarrhoea, fever, immune system booster, infections, oedema and ulcers                                                                                                                                                                                                             | 3                  | [25,26,39]                |
| * <i>Cajanus cajan</i> (L.) Huth. <sup>1</sup>                         | Shrub | Leaves                        | Earache                                                                                                                                                                                                                                                                                                                                   | 1                  | [25]                      |
| <i>Cassia abbreviata</i> Oliv. <sup>2</sup>                            | Tree  | Bark, fruits, roots and twigs | Abdominal pains, abortifacient, aphrodisiac, backache, bilharzia, cancer, constipation, diarrhoea, gonorrhoea, hydrocele, lucky charms, malaria, menstrual problems, stomach pains, STDs and venereal diseases                                                                                                                            | 11                 | [25–28,34–38,40–42]       |
| <i>Colophospermum mopane</i> (J.Kirk ex Benth.) J.Léonard <sup>2</sup> | Tree  | Bark, charcoal, leaves        | Constipation, diarrhoea, snake bite and diarrhoea in cattle                                                                                                                                                                                                                                                                               | 3                  | [25,28,38]                |
| <i>Crotalaria laburnifolia</i> L. <sup>1</sup>                         | Herb  | Roots                         | Cough                                                                                                                                                                                                                                                                                                                                     | 1                  | [25]                      |
| <i>Crotalaria rogersii</i> Bak.f. <sup>1</sup>                         | Herb  | Roots                         | Infertility in women and lucky charms                                                                                                                                                                                                                                                                                                     | 1                  | [25]                      |
| <i>Dalbergia melanoxylon</i> Guill. & Perr. <sup>1</sup>               | Shrub | Bark                          | Asthma and wounds                                                                                                                                                                                                                                                                                                                         | 3                  | [37,38,43]                |
| <i>Dalbergia nitidula</i> Welw. ex Bak. <sup>1</sup>                   | Shrub | Bark and roots                | Aphrodisiac, driving away bad spirits, preventing witchcraft and ulcers                                                                                                                                                                                                                                                                   | 1                  | [25]                      |
| <i>Dalbergiella nyasae</i> Bak.f. <sup>1</sup>                         | Tree  | Leaves and roots              | Tetanic contractions and driving away maggots from wounds                                                                                                                                                                                                                                                                                 | 1                  | [25]                      |
| <i>Dichrostachys cinerea</i> (L.) Wight & Arn. <sup>2</sup>            | Tree  | Leaves, fruits or roots       | Abdominal pains, antivenom, backache, cancer, colic, contraceptive, cough, depressed fontanelle, diarrhoea, dilating the birth canal, epistaxis, infertility in women, influenza, inducing labour, mental problems, oedema, postpartum, scabies, scorpion stings, STDs, stomach problems, syphilis, urticaria (skin swellings) and wounds | 8                  | [25,28,29,31,34,35,38,40] |
| <i>Dolichos kilimandscharicus</i> Taub. <sup>1</sup>                   | Herb  | Tubers                        | Abdominal pains, antiemetic, constipation, diarrhoea and measles                                                                                                                                                                                                                                                                          | 1                  | [25]                      |
| <i>Elephantorrhiza burkei</i> Benth. <sup>2</sup>                      | Shrub | Roots                         | Antiemetic, constipation, increasing blood in the body and postpartum conditions                                                                                                                                                                                                                                                          | 1                  | [25]                      |

Table 1. Cont.

| Plant Taxa                                                            | Habit | Parts Used              | Medicinal Uses                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Literature Records | References          |
|-----------------------------------------------------------------------|-------|-------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|---------------------|
| <i>Elephantorrhiza elephantina</i> (Burch.) Skeels <sup>2</sup>       | Shrub | Roots                   | Abdominal pains, aphrodisiac, infertility in women, postpartum conditions and reducing the size of the vagina                                                                                                                                                                                                                                                                                                                                                                    | 3                  | [25,34,44]          |
| <i>Elephantorrhiza goetzei</i> (Harms) Harms <sup>2</sup>             | Shrub | Bark, rhizomes or roots | Abdominal pains, anthelmintic, backache, bilharzia, bloating, blood pressure, boosting appetite, constipation, cough, depressed fontanelle, diarrhoea, dilating the birth canal, erectile function, fever, gonorrhoea, heart pains, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV / AIDs) opportunistic infections, increasing blood in the body, infertility, influenza, malaria, painful uterus, postpartum conditions, rash, STIs and stomach problems | 11                 | [25–28,31,34–38,41] |
| <i>Elephantorrhiza suffruticosa</i> Schinz <sup>2</sup>               | Shrub | Roots                   | Constipation and diarrhoea                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 1                  | [25]                |
| <i>Eriosema englerianum</i> Harms <sup>1</sup>                        | Shrub | Leaves and roots        | Aphrodisiac, backache, bilharzia, blood pressure, infertility in women, menstrual problems, painful uterus, venereal disease and wasting in infants                                                                                                                                                                                                                                                                                                                              | 3                  | [25,27,31]          |
| <i>Eriosema rhynchosioides</i> Bak. <sup>1</sup>                      | Shrub | Roots                   | Tonic                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 1                  | [25]                |
| <i>Erythrina abyssinica</i> Lam. ex DC. <sup>1</sup>                  | Tree  | Bark, leaves and roots  | Abdominal pains, backache, bilharzia, blood pressure, cough, cracked heels, diarrhoea, gonorrhoea, lucky charms, mental problems, STDs, stop bad dreams, wasting in infants, wounds and wounds in the mouth                                                                                                                                                                                                                                                                      | 9                  | [25–29,31,36,37,40] |
| <i>Erythrina livingstoniana</i> Bak. <sup>1</sup>                     | Tree  | Roots                   | Haematuria                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 1                  | [25]                |
| <i>Erythrina</i> spp. <sup>1</sup>                                    |       | Roots                   | Backache and manic disorders                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 1                  | [29]                |
| <i>Erythrophileum africanum</i> (Benth.) Harms <sup>2</sup>           | Tree  | Bark                    | Stomach pains                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 1                  | [25]                |
| <i>Erythrophileum suaveolens</i> (Guill. & Perr.) Brenan <sup>2</sup> | Tree  | Bark                    | Preventing witchcraft                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 1                  | [25]                |
| <i>Flemingia grahamiana</i> Wight & Arry <sup>1</sup>                 | Herb  | Roots                   | Diarrhoea                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 1                  | [25]                |
| <i>Grona barbata</i> (L.) H.Ohashi & K.Ohashi <sup>1</sup>            | Herb  | Roots                   | Abortifacient, dilating the birth canal, epilepsy, preventing abortion, postpartum conditions, sore eyes and wasting in infants                                                                                                                                                                                                                                                                                                                                                  | 1                  | [25]                |
| <i>Indigofera antunesiana</i> Harms <sup>1</sup>                      | Shrub | Roots                   | Menstrual problems                                                                                                                                                                                                                                                                                                                                                                                                                                                               | 1                  | [25]                |
| <i>Indigofera arrecta</i> Hochst. ex A.Rich. <sup>1</sup>             | Shrub | Leaves and roots        | Abdominal pains, abortifacient, convulsions, diuretic, gonorrhoea, infertility, purgative, sore eyes, stomach pains and de-ticking dogs                                                                                                                                                                                                                                                                                                                                          | 2                  | [25,35]             |



Table 1. Cont.

| Plant Taxa                                                                                                                   | Habit   | Parts Used                         | Medicinal Uses                                                                                                           | Literature Records | References |
|------------------------------------------------------------------------------------------------------------------------------|---------|------------------------------------|--------------------------------------------------------------------------------------------------------------------------|--------------------|------------|
| <i>Indigofera astragalina</i> DC. <sup>1</sup>                                                                               | Herb    | Roots                              | Dizziness                                                                                                                | 1                  | [25]       |
| <i>Indigofera demissa</i> Taub. <sup>1</sup>                                                                                 | Herb    | Roots                              | Abortifacient                                                                                                            | 1                  | [25]       |
| <i>Indigofera hilaris</i> Eckl. & Zeyh. <sup>1</sup>                                                                         | Herb    | Roots                              | Painful legs                                                                                                             | 1                  | [25]       |
| <i>Indigofera hirsuta</i> L. <sup>1</sup>                                                                                    | Shrub   | Roots                              | Dizziness                                                                                                                | 1                  | [34]       |
| <i>Indigofera rhynchocarpa</i> Bak. <sup>1</sup>                                                                             | Shrub   | Roots                              | Abdominal pains and menstrual problems                                                                                   | 1                  | [25,34]    |
| <i>Indigofera setiflora</i> Baker <sup>1</sup>                                                                               | Herb    | Roots                              | Diarrhoea and stomach problems                                                                                           | 3                  | [28,37,43] |
| <i>Indigofera spicata</i> Forssk. <sup>1</sup>                                                                               | Herb    | Roots                              | Panacea                                                                                                                  | 1                  | [25]       |
| <i>Indigofera vicinoides</i> Jaub. & Spach. ssp. <i>rogersii</i> (R.E.Fr.) Schrire <sup>1</sup>                              | Shrub   | Roots                              | Depressed fontanelle                                                                                                     | 1                  | [25]       |
| <i>Indigofera wilsonii</i> J.B.Gillett <sup>1</sup>                                                                          | Shrub   | Roots                              | Preventing abortion                                                                                                      | 1                  | [25]       |
| <i>Indigofera</i> spp. <sup>1</sup>                                                                                          | Shrub   | Leaves and roots                   | Abdominal pains, antenatal conditions, chest pains, coughs, driving away bad spirits and infertility in women            | 2                  | [25,34]    |
| <i>Julbernardia globiflora</i> (Benth.) Troupin <sup>2</sup>                                                                 | Tree    | Bark, bark fibre, leaves and roots | Constipation, diarrhoea, reducing the size of the vagina, snakebite, sore eyes, stomach problems and diarrhoea in cattle | 3                  | [25,28,38] |
| * <i>Lessertia frutescens</i> (L.) Goldblatt & J.C.Manning <sup>1</sup> (Syn. <i>Sutherlandia frutescens</i> (L.) W.T.Aiton) | Shrub   | Roots                              | Analgesia, cancer, colds, diabetes, fever, influenza and haemorrhoids                                                    | 1                  | [39]       |
| * <i>Leucaena leucocephala</i> (Lam.) DeWit <sup>2</sup>                                                                     | Shrub   | Bark, leaves and seeds             | Colds, influenza and TB                                                                                                  | 1                  | [45]       |
| <i>Macrotyloma densiflorum</i> (Welw. ex Bak.) Verdc. <sup>1</sup>                                                           | Shrub   | Leaves                             | Abdominal pains                                                                                                          | 1                  | [25]       |
| <i>Mucuna coriacea</i> Baker <sup>1</sup>                                                                                    | Climber | Roots                              | Bilharzia                                                                                                                | 1                  | [26]       |
| <i>Mundulea sericea</i> (Willd.) A.Chev. <sup>1</sup>                                                                        | Shrub   | Roots                              | Infertility and sexual impotence                                                                                         | 2                  | [25,29]    |
| <i>Neorautanenia mitis</i> (A.Rich.) Verdc. <sup>1</sup>                                                                     | Climber | Bulbs                              | Fever and de-ticking dogs                                                                                                | 2                  | [25,28]    |
| <i>Ormocarpum kirkii</i> S.Moore <sup>1</sup>                                                                                | Tree    | Leaves                             | Depressed fontanelle, dilating the birth canal and stomach pains                                                         | 3                  | [28,31,34] |
| <i>Ormocarpum trichocarpum</i> (Taub.) Engl. <sup>1</sup>                                                                    | Shrub   | Leaves and roots                   | Allergies, depressed fontanelle, prolonged labour and stomach problems                                                   | 2                  | [25,29]    |

Table 1. Cont.

| Plant Taxa                                                                                                     | Habit   | Parts Used                                   | Medicinal Uses                                                                                                                                                                                                                                                                                                                                | Literature Records | References          |
|----------------------------------------------------------------------------------------------------------------|---------|----------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|---------------------|
| <i>Peltophorum africanum</i> Sond. <sup>2</sup>                                                                | Tree    | Bark, leaves and roots                       | Abdominal pains, bilharzia, blood purification, chest pains, diaphoretic, diarrhoea, diuretic, driving away evil spirits, dropsy, eye problems, headache, infertility in women, laxative, mental problems, nausea, oedema, panacea, preventing abortion, sore eyes, sore throat, STDs, STIs, syphilis, toothache and venereal diseases        | 10                 | [25–27,29,34–38,46] |
| <i>Pericopsis angolensis</i> (Baker) Meeuwen <sup>1</sup>                                                      | Tree    | Bark or roots                                | Abdominal pains, antiemetic, backache, cancer, cough, diarrhoea, dyspnoea, oedema, sore throats and wounds                                                                                                                                                                                                                                    | 3                  | [25,28,38]          |
| * <i>Phaseolus vulgaris</i> L. <sup>2</sup>                                                                    | Shrub   | Roots                                        | Bilharzia and postpartum conditions                                                                                                                                                                                                                                                                                                           | 2                  | [26,34]             |
| <i>Philenoptera violacea</i> (Klotzch) Schrire <sup>1</sup>                                                    | Tree    | Roots                                        | Diarrhoea                                                                                                                                                                                                                                                                                                                                     | 1                  | [38]                |
| <i>Ptilostigma thonningii</i> (Schumach.) Milne-Redh. <sup>2</sup> (Syn. <i>Baulinia thonningii</i> Schumach.) | Tree    | Bark, fruits, leaves and roots               | Abdominal pains, antivenom, bilharzia, constipation, convulsions, cough, diarrhoea, dropsy, emetic, immune booster, influenza, menstrual problems, painful legs, painful uterus, postpartum conditions, stomach problems and ketosis in cattle                                                                                                | 7                  | [25,26,28,29,36–38] |
| <i>Pseudarthria hookeri</i> Wight & Arn. <sup>1</sup>                                                          | Herb    | Leaves and roots                             | Bilharzia and diarrhoea                                                                                                                                                                                                                                                                                                                       | 1                  | [25]                |
| <i>Pterocarpus angolensis</i> DC. <sup>1</sup>                                                                 | Tree    | Bark, flowers, fruits, leaves, roots and sap | Abdominal pains, anaemia, aphrodisiac, asthma, backache, bilharzia, body pains, cataract, cough, depressed fontanelle, diarrhoea, earache, haematuria, infertility in women, kwashiorkor, lameness, menstrual problems, pelvic inflammation, ringworm, sore eyes, stomach problems, TB, ulcers and venereal diseases and sore eyes in animals | 11                 | [25–29,31,35–38,46] |
| <i>Pterocarpus rotundifolius</i> (Sond.) Druce <sup>1</sup>                                                    | Tree    | Root sap                                     | Sore eyes                                                                                                                                                                                                                                                                                                                                     | 1                  | [25]                |
| <i>Pterolobium stellatum</i> (Forssk.) Brenan <sup>2</sup>                                                     | Climber | Roots                                        | Augmenting labour and depressed fontanelle                                                                                                                                                                                                                                                                                                    | 2                  | [31,38]             |
| <i>Rhynchosia insignis</i> (O.Hoffm.) R.E.Fr. <sup>1</sup>                                                     | Herb    | Roots                                        | Abdominal pains, depressed fontanelle and dropsy                                                                                                                                                                                                                                                                                              | 1                  | [25]                |
| <i>Rhynchosia minutina</i> (L.) DC. <sup>1</sup>                                                               | Herb    | Roots                                        | Boils and skin infections                                                                                                                                                                                                                                                                                                                     | 1                  | [47]                |
| <i>Rhynchosia monophylla</i> Schltr. <sup>1</sup>                                                              | Herb    | Roots                                        | Postpartum conditions                                                                                                                                                                                                                                                                                                                         | 1                  | [25]                |
| <i>Rhynchosia resinosa</i> (Hochst. ex A.Rich.) Bak. <sup>1</sup>                                              | Climber | Leaves and roots and twigs                   | Abdominal pains, diabetes mellitus, dilating the birth canal, expel maggots from wounds, high blood pressure, infertility and menstrual problems                                                                                                                                                                                              | 3                  | [25,31,34]          |
| <i>Rhynchosia</i> spp. <sup>1</sup>                                                                            | Herb    | Roots                                        | Diarrhoea                                                                                                                                                                                                                                                                                                                                     | 1                  | [38]                |

Table 1. Cont.

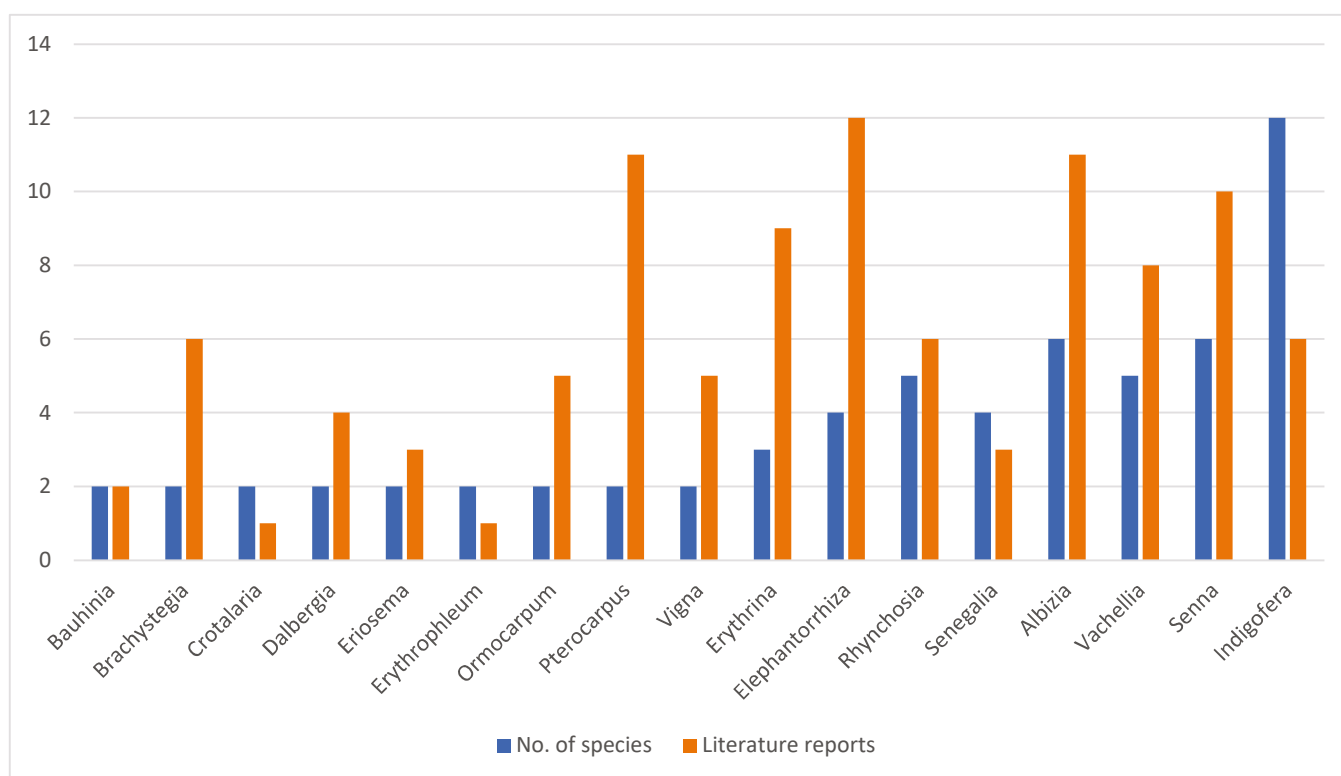
| Plant Taxa                                                                                                                                                | Habit | Parts Used             | Medicinal Uses                                                                                                                                                                                                                                           | Literature Records | References       |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------|-------|------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|------------------|
| <i>Schotia brachypetala</i> Sond. <sup>2</sup>                                                                                                            | Tree  | Bark, leaves and roots | Depressed fontanelle, diarrhoea, dysentery, epistaxis, oedema, stomach problems, swellings and ulcers                                                                                                                                                    | 4                  | [25,28,34,38]    |
| <i>Senegalia ataxacantha</i> (DC.) Kyal. & Boatwr. <sup>2</sup><br>(Syn. <i>Acacia ataxacantha</i> DC.)                                                   | Shrub | Roots                  | Abdominal pains, constipation and preventing witchcraft                                                                                                                                                                                                  | 1                  | [25]             |
| <i>Senegalia chariessa</i> (Milne-Redh.) Kyal. & Boatwr. <sup>2</sup><br>(Syn. <i>Acacia chariessa</i> Milne-Redh.)                                       | Shrub | Roots                  | Antenatal, blood purification and postpartum                                                                                                                                                                                                             | 2                  | [25,29]          |
| <i>Senegalia mellifera</i> (Benth.) Seigler & Ebinger <sup>2</sup><br>(Syn. <i>Acacia mellifera</i> Benth.)                                               | Shrub | Bark                   | Aphrodisiac                                                                                                                                                                                                                                              | 2                  | [25,28]          |
| <i>Senegalia nigrescens</i> (Oliv.) P.J.H.Hurter <sup>2</sup><br>(Syn. <i>Acacia nigrescens</i> Oliv.)                                                    | Tree  | Roots                  | Convulsions                                                                                                                                                                                                                                              | 1                  | [25]             |
| <i>Senna didymobotrya</i> (Fresen.) H.S.Irwin & Barneby <sup>2</sup><br>(Syn. <i>Cassia didymobotrya</i> Fresen.)                                         | Shrub | Roots                  | Convulsions and mental problems                                                                                                                                                                                                                          | 1                  | [25]             |
| <i>Senna italica</i> Mill. <sup>2</sup><br>(Syn. <i>Cassia italica</i> (Mill.) F.W.Andr.)                                                                 | Shrub | Roots                  | Abdominal pains, bilharzia, bronchitis, colic, haemorrhoids and sore eyes                                                                                                                                                                                | 3                  | [25,26,29]       |
| * <i>Senna occidentalis</i> (L.) Link <sup>2</sup><br>(Syn. <i>Cassia occidentalis</i> L.)                                                                | Herb  | Roots                  | Sore throats and tonsillitis                                                                                                                                                                                                                             | 1                  | [28]             |
| <i>Senna petersiana</i> (Bolle) Lock <sup>2</sup><br>(Syn. <i>Cassia petersiana</i> Bolle)                                                                | Tree  | Roots                  | Bilharzia                                                                                                                                                                                                                                                | 1                  | [26]             |
| <i>Senna singuana</i> (Delile) Lock <sup>2</sup><br>(Syn. <i>Cassia singuana</i> Delile)                                                                  | Shrub | Bark, leaves and roots | Abdominal pains, antiemetic, bilharzia, constipation, dropsy, herpes, infertility in women, malaria, menstrual problems, painful uterus, postpartum, preventing still birth, preventing bad luck, sores, sore eyes, STDs, syphilis and venereal diseases | 7                  | [25–28,34,38,40] |
| * <i>Senna septentrionalis</i> (Viv.) H.S.Irwin & Barneby <sup>2</sup> (Syn. <i>Cassia septentrionalis</i> Viv.)                                          | Shrub | Roots                  | Malaria                                                                                                                                                                                                                                                  | 1                  | [41]             |
| <i>Sesbania</i> spp. <sup>1</sup>                                                                                                                         | Shrub | Seeds                  | Fever                                                                                                                                                                                                                                                    | 1                  | [34]             |
| <i>Sphenostylis erecta</i> (Baker f.) Hutch. ex Baker f. <sup>1</sup><br>(Syn. <i>Sphenostylis marginata</i> E.Mey. ssp. <i>erecta</i> (Baker f.) Verdc.) | Shrub | Roots                  | Abdominal pains, bile emesis, constipation, diarrhoea, fever, oedema and wasting away in infants                                                                                                                                                         | 2                  | [25,38]          |
| * <i>Tamarindus indica</i> L. <sup>2</sup>                                                                                                                | Tree  | Fruits and roots       | Sore throat and venereal diseases                                                                                                                                                                                                                        | 1                  | [25]             |

Table 1. Cont.

| Plant Taxa                                                                                                                                  | Habit   | Parts Used             | Medicinal Uses                                                                                                                                                                                                                        | Literature Records | References       |
|---------------------------------------------------------------------------------------------------------------------------------------------|---------|------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|------------------|
| <i>Tephrosia radicans</i> Welw. <sup>1</sup>                                                                                                | Shrub   | Roots                  | Sore eyes and toothache                                                                                                                                                                                                               | 1                  | [25]             |
| <i>Tylosema fassoglense</i> (Kotschy ex Schweinf.) Torre & Hillc. <sup>2</sup>                                                              | Climber | Bark, roots and tubers | Abdominal pains, diarrhoea, pneumonia, retained placenta, stomach problems and venereal diseases                                                                                                                                      | 2                  | [25,28]          |
| <i>Vachellia amygdaliophylla</i> (Steud. ex A.Rich.) Kyal. & Boatwr. <sup>2</sup><br>(Syn. <i>Acacia amygdaliophylla</i> Steud. ex A.Rich.) | Shrub   | Roots                  | Antidote for snakebites, convulsions, driving away evil spirits, excessive sweating, infertility in women, mental problems and painful uterus                                                                                         | 1                  | [25]             |
| <i>Vachellia karroo</i> (Hayne) Banfi & Galasso <sup>2</sup><br>(Syn. <i>Acacia karroo</i> Hayne)                                           | Tree    | Fruits and roots       | Aphrodisiac, bilharzia, body pains, convulsions, dizziness, gonorrhoea, syphilis and killing parasites in fowl runs                                                                                                                   | 6                  | [25–27,36,37,43] |
| <i>Vachellia nilotica</i> (L.) P.J.H.Hurter & Mabb. <sup>2</sup><br>(Syn. <i>Acacia nilotica</i> L.)                                        | Shrub   | Fruits and roots       | STDs                                                                                                                                                                                                                                  | 1                  | [40]             |
| <i>Vachellia rehmanniana</i> (Schinz) Kyal. & Boatwr. <sup>2</sup><br>(Syn. <i>Acacia rehmanniana</i> Schinz)                               | Tree    | Bark and roots         | Bloated stomach, headaches and pneumonia                                                                                                                                                                                              | 1                  | [25,28]          |
| <i>Vachellia sieberiana</i> (DC.) Kyal. & Boatwr. <sup>2</sup><br>(Syn. <i>Acacia sieberiana</i> DC.)                                       | Shrub   | Roots                  | Antiseptic                                                                                                                                                                                                                            | 1                  | [25]             |
| <i>Vigna nuda</i> N.E.Br. <sup>1</sup>                                                                                                      | Herb    | Roots                  | Chest pains and cough                                                                                                                                                                                                                 | 1                  | [34]             |
| <i># Vigna unguiculata</i> (L.) Walp. <sup>1</sup>                                                                                          | Shrub   | Roots and seeds        | Anaemia, antivenom, bilharzia, chest pains, constipation, epilepsy, menstrual problems and antidote for snakebites                                                                                                                    | 4                  | [25–28]          |
| <i>Xeroderris stuhlmannii</i> (Taub.) Mendonça & E.P.Sousa <sup>1</sup>                                                                     | Tree    | Bark, leaves or roots  | Abdominal pains, anaemia, antiabortifacient, back pains, cancer, diarrhoea, headache, infertility in men, malaria, menstrual problems, pneumonia, stomach problems, toothache, venereal diseases, wounds and ethnoveterinary medicine | 4                  | [25,28,38,48]    |
| <i>Zornia gluchidiata</i> Rchb. ex DC. <sup>1</sup>                                                                                         | Herb    | Roots                  | Dilating the birth canal, preventing abortion and venereal diseases                                                                                                                                                                   | 1                  | [25]             |

\* = Exotic; # = cultivated or collected from semi-natural landscapes; <sup>1</sup> = Faboideae, <sup>2</sup> = Caesalpinioideae, <sup>3</sup> = Cercidoideae.

*Indigofera* is the genus with the highest number of medicinal Fabaceae species (12 species), followed by *Senna* (six species), and *Albizia*, *Rhynchosia* and *Vachellia* with five species each (Figure 2). However, the genera associated with the highest number of records in the literature are *Elephantorrhiza* (12 records), *Pterocarpus* (11 records), *Senna* (10 records), *Albizia* and *Erythrina* (nine records each) and *Vachellia* with eight records (Figure 2). The number of medicinal species found in each genus is significantly correlated to the total number of species in each genus in Zimbabwe ( $p < 0.01$ ,  $r = 0.772$ ). These results are consistent with those observed by Anorl et al. [49], who recorded 11 medicinal species of *Albizia*, followed by *Rhynchosia* (12 species), *Senna* (17 species), and *Indigofera* and *Vachellia* with 32 species each. In Botswana, Hedberg and Staugård [50] argued that one *Senna* species, followed by *Albizia* (three species), *Rhynchosia* (four species), *Indigofera* (five species) and *Vachellia* (six species) were used as traditional medicines in that country. Moreover, several species of *Albizia*, *Elephantorrhiza*, *Erythrina*, *Senna* and *Vachellia* are included in the monograph *Medicinal Plants of South Africa*, with detailed information on their botany, medicinal uses, preparation, dosage, active ingredients and pharmacological effects [51].

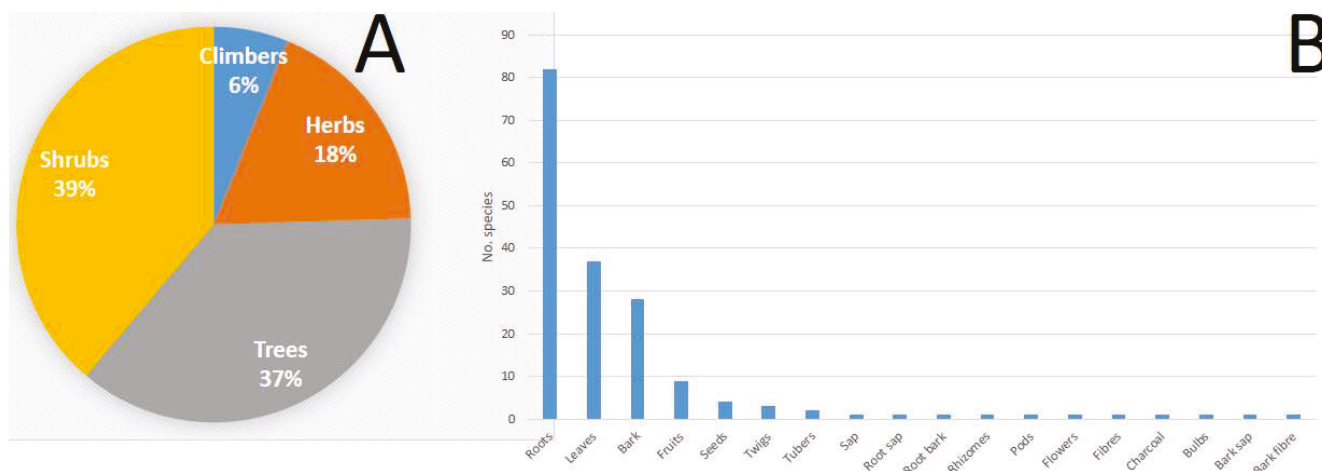


**Figure 2.** Numbers of species and reports of the use of Fabaceae genera with medicinal uses in Zimbabwe.

### 3.2. Growth Habit and Parts Used

Shrubs (39.0%), followed by trees (37.0%) and herbs (18.0%), are the primary sources of the medicinal Fabaceae species in Zimbabwe (Figure 3A). The plant parts used for traditional medicine preparations include bark, bark fibre, bark sap, bulbs, charcoal, fibre, flowers, fruits, leaves, pods, rhizomes, roots, root bark, root sap, sap, seeds, tubers and twigs (Table 1). The roots are the most frequently used (81 species), followed by leaves (37 species), bark (28 species), fruits (nine species), seeds (four species), twigs (three species) and tubers (two species), with the rest of the plant parts represented by a single species each (Figure 3B). However, harvesting the roots of herbaceous plants for medicinal purposes is not sustainable, as it threatens the survival of these plants used to treat human and animal diseases. It is well recognized by conservationists that medicinal plants primarily

valued for their roots and those which are intensively harvested for their bark often tend to be the most threatened by overexploitation [52,53]. *Afzelia quanzensis*, *Baikiaea plurijuga*, *Dalbergia melanoxylon* and *Pterocarpus angolensis* are listed in the Zimbabwean Red Data List, as these four species are threatened with extinction mainly due to overexploitation as sources of timber for construction or wood carving [54].



**Figure 3.** Characteristics of Fabaceae species used as traditional medicines in Zimbabwe. (A): Growth habit as a pie diagram and (B): Plant parts used presented as a bar chart.

### 3.3. Usage Categories with High Numbers of Reports

The 134 medical reports of Fabaceae species in Zimbabwe (Tables 1 and 2) are classified into 19 major health disorder categories following the International Classification of Primary Care's classification system [19]. Most use records are in the categories of gastrointestinal problems (92 usage reports) and female reproductive problems (58 usage reports) (Table 2). Similarly, gastrointestinal problems, reproductive problems in women, respiratory problems and sexually transmitted infections (Table 2) are treated with the highest number of species. The categories of gastrointestinal problems, reproductive problems, respiratory problems and sexually transmitted infections are among the 10 major causes of death in Zimbabwe [55]. Muchandiona [56] argued that the prevalence of gastrointestinal disorders and respiratory infections is due to poor solid waste management by the local councils in Zimbabwe, which has worsened over the years. Similarly, gastrointestinal disorders, such as diarrhoea and dysentery, are also a major concern in neighbouring countries such as Mozambique [57–59] and South Africa [60–62]. Therefore, gastrointestinal problems are among the most common reasons local people use traditional medicines and consult traditional healers [57,59–62].

**Table 2.** Major disease categories and Fabaceae species used as traditional medicines in Zimbabwe.

| Disease Category                    | Species | Usage Records |
|-------------------------------------|---------|---------------|
| Antenatal and postpartum conditions | 15      | 23            |
| Antivenom                           | 8       | 11            |
| Back pain                           | 10      | 14            |
| Bilharzia                           | 19      | 27            |
| Charms and ritual objects           | 15      | 26            |
| Convulsions and epilepsy            | 9       | 18            |
| Depressed fontanelle                | 12      | 24            |
| Ethnoveterinary medicine            | 10      | 20            |

Table 2. Cont.

| Disease Category                | Species | Usage Records |
|---------------------------------|---------|---------------|
| Fever and malaria               | 11      | 19            |
| Gastrointestinal problems       | 45      | 92            |
| Mental problems                 | 10      | 16            |
| Oedema                          | 11      | 19            |
| Reproductive problems in men    | 18      | 36            |
| Reproductive problems in women  | 27      | 58            |
| Respiratory problems            | 26      | 39            |
| Sexually transmitted infections | 20      | 31            |
| Skin problems                   | 8       | 16            |
| Sore eyes                       | 15      | 27            |
| Sores and wounds                | 11      | 24            |

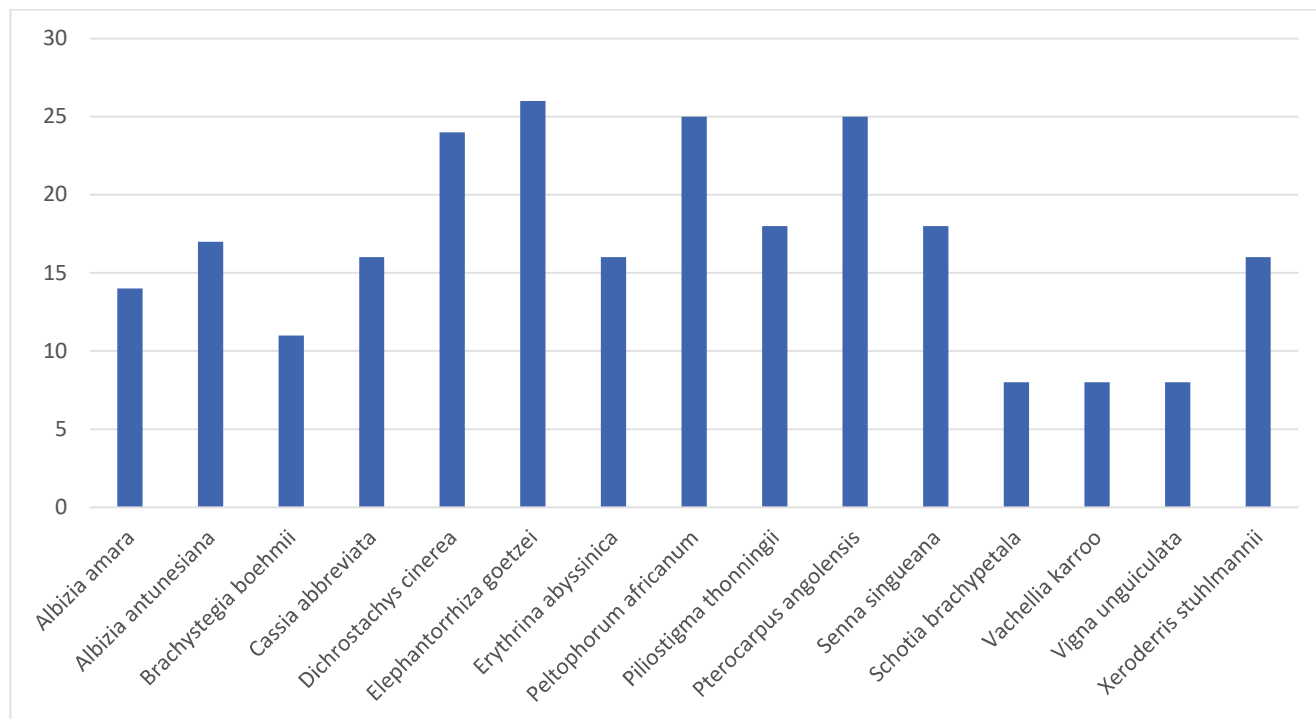
Fifteen medicinal species are known to have more than eight usage reports (Figure 4). These species included *Albizia amara*, *Albizia antunesiana*, *Brachystegia boehmii*, *Cassia abbreviate*, *Dichrostachys cinerea*, *Elephantorrhiza goetzei*, *Erythrina abyssinica*, *Peltophorum africanum*, *Piliostigma thonningii*, *Pterocarpus angolensis*, *Schotia brachypetala*, *Senna singueana*, *Vachellia karroo*, *Vigna unguiculata* and *Xeroderris stuhlmannii*. Some of these plant species are widely used as sources of traditional medicines in Angola [63], Botswana [50,64], Eswatini [65], Malawi [66,67], Mozambique [58,68], Namibia [69,70], South Africa [71,72] and Zambia [73,74]. The importance of these species as sources of traditional medicines is documented in the monographs *Medicinal and Magical Plants of Southern Africa: An Annotated Checklist* [49], *Plant Resources of Tropical Africa 11: Medicinal Plants 1 and 2* [75,76] and *Medicinal Plants of South Africa* [51]. Research by Van Wyk [77] revealed that *Colophospermum mopane*, *Dichrostachys cinerea* and *Vachellia karroo* are commercially exploited in local, regional or international trade in eastern, southern and western Africa.

### 3.4. Phytochemistry and Pharmacological Properties of Fabaceae Species

The Fabaceae species used as sources of traditional medicines in Zimbabwe are rich in chemical constituents (Table 3). The majority of these species are characterized by flavonoids (57.4%), followed by terpenoids (42.6%), tannins (40.6%), saponins (34.7%), phenolics (30.7%) and alkaloids (28.7%) (Table 3). Research by Wink [78] showed that the main secondary metabolites of the Fabaceae family include alkaloids, non-protein amino acids, cyanogens, peptides, phenolics, polyketides and terpenoids. This author argued that these secondary metabolites serve as defence compounds against herbivores and microbes and also serve as signal compounds to attract pollinating and fruit-dispersing animals. Fabaceae species used as traditional medicines and food plants are characterized by nutrients such as proteins, lipids, carbohydrates, mineral elements, fatty acids, amino acids, fibres and vitamins, which are important for animal and human health [79,80]. The majority of documented species have several proven pharmacological activities (Table 3) such as inhibition of the acetylcholinesterase enzyme, and anticancer, antidiabetic, antifertility, anthelmintic, antiamoebic, anti-inflammatory, antimicrobial, antioxidant, antiparasitic, cytotoxic, hepatoprotective, hypoglycaemic and immunomodulatory effects. Despite the discovery of several secondary metabolites in the Fabaceae family, its species have attracted disproportionately little attention in the context of ethnopharmacological research over the years. The relative importance of the Fabaceae species as medicinal plants is demonstrated by the fact that about 10% of the species documented in this study are commercially important. The species that are commercially developed with potential to be developed into health products or pharmaceutical drugs and are regularly traded on the international



markets include *Abrus precatorius*, *Albizia adianthifolia*, *Cajanus cajan*, *Colophospermum mopane*, *Dichrostachys cinerea*, *Lessertia frutescens*, *Senna italica*, *Senna occidentalis*, *Tamarindus indica*, *Vachellia karroo* and *Vachellia nilotica* [77,81].



**Figure 4.** Fabaceae species with eight or more usage reports cited in at least four references.

**Table 3.** Phytochemistry and pharmacological properties of Fabaceae species used as traditional medicines in Zimbabwe.

| Species                         | Phytochemistry                                                                                                                                                                        | Pharmacological Activities                                                                                                                                                  | References |
|---------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>Abrus precatorius</i>        | Alkaloids, esters, flavonoids, organic acids, phenolics, steroids and terpenoids                                                                                                      | Antidiabetic, antifertility, anti-inflammatory, antimicrobial, antioxidant, antiparasitic, antiprotozoal, antitumor, immunomodulatory and insecticidal                      | [82]       |
| <i>Aeschynomene mimosifolia</i> | Flavonoids                                                                                                                                                                            | Cytotoxicity                                                                                                                                                                | [83]       |
| <i>Azelia quanzensis</i>        | Fatty acids                                                                                                                                                                           | Antifungal                                                                                                                                                                  | [84]       |
| <i>Albizia amara</i>            | Alkaloids, glycosides, flavonoids, phenols, quinones, saponins, sterols, tannins and terpenoids                                                                                       | Analgesic, antiarthritic, antibacterial, antifungal, antiviral, anticancer, antihyperlipidemic, anti-inflammatory, antioxidant and hepatoprotective                         | [85–87]    |
| <i>Albizia adianthifolia</i>    | Apocarotenoids, chalcone, dipeptide, elliptocytes, fatty acids, flavonoids, histamine, imidazolyl carboxylic acid, prosapogenins, saponins, steroids, triterpenoids and volatile oils | Acetylcholinesterase enzyme inhibitory, anthelmintic, antiamoebic, antibacterial, antifungal, anti-inflammatory, antioxidant, cytotoxic, hypoglycaemic and immunomodulatory | [88,89]    |
| <i>Albizia anthelmintica</i>    | Alkaloids, diterpenes, flavonoids, gallic acid, phenolics, saponins and tannins                                                                                                       | Analgesic, antibacterial, anti-inflammatory and antioxidant                                                                                                                 | [90,91]    |
| <i>Albizia antunesiana</i>      | Coumarins, phenolics and triterpenoids                                                                                                                                                | Anthelmintic and antioxidant                                                                                                                                                | [92]       |
| <i>Albizia tanganyicensis</i>   | Saponins                                                                                                                                                                              | Anthelmintic, anticonvulsant, anti-inflammatory, antimicrobial, antioxidant and wound healing                                                                               | [93–95]    |



Table 3. Cont.

| Species                            | Phytochemistry                                                                                                                       | Pharmacological Activities                                                                                                                                | References   |
|------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|
| <i>Albizia versicolor</i>          | Glycosides, saponins and triterpenes                                                                                                 | Anthelmintic and antifungal                                                                                                                               | [84,96,97]   |
| <i>Arachis hypogaea</i>            | Alkaloids, phenolics, phytic acid and saponins                                                                                       | Antioxidant                                                                                                                                               | [98]         |
| <i>Baikiaea plurijuga</i>          | Alkaloids, anthraquinones, flavonoids, phenolics and tannins                                                                         | Antibacterial and antioxidant                                                                                                                             | [99–101]     |
| <i>Bauhinia galpinii</i>           | Fatty acids, flavonoids, phenols, proanthocyanidin, tannins and terpenoids                                                           | Antibacterial, antifungal, anti-inflammatory, antioxidant and cytotoxic                                                                                   | [102–106]    |
| <i>Bauhinia petersiana</i>         | Anthraquinones, alkaloids, cardenolides, flavonoids, saponins, tannins and terpenoids                                                | Antibacterial, antifungal, anti-inflammatory, antioxidative and cytotoxic                                                                                 | [102,104]    |
| <i>Bobgunnia madagascariensis</i>  | Flavonoids, saponins and tannins                                                                                                     | Antibacterial                                                                                                                                             | [107]        |
| <i>Bolusanthus speciosus</i>       | Alkaloids, flavonoids, phenolics, saponins, tannins and volatile oils                                                                | Anti-arthritis, antibacterial, antigonococcal, antimycobacterial, antifungal, anti-HIV, anti-inflammatory and antioxidant                                 | [108]        |
| <i>Brachystegia boehmii</i>        | Tannins                                                                                                                              | Antibacterial, anti-inflammatory and antioxidant                                                                                                          | [109–111]    |
| <i>Brachystegia spiciformis</i>    | Proanthocyanadin and tannins                                                                                                         | * None found                                                                                                                                              | [112,113]    |
| <i>Burkea africana</i>             | Flavonoids, glycosides, saponins, steroids, tannins and triterpenes                                                                  | Analgesic, antibacterial, antiviral, anticholinesterase, anti-inflammatory and antioxidant                                                                | [68,114–116] |
| <i>Cajanus cajan</i>               | Coumarins, flavonoids, phenolics and stilbenes                                                                                       | Antioxidant and anti-inflammatory                                                                                                                         | [117]        |
| <i>Cassia abbreviata</i>           | Anthocyanins, anthranoids, anthraquinones, polyphenols and tannins                                                                   | Abortifacient, anti-diabetic, anti-inflammatory, antimicrobial, antiviral, antioxidant and hepatoprotective                                               | [118–120]    |
| <i>Colophospermum mopane</i>       | Alkaloids, coumarins, diterpenes, flavonoids, polyphenols, proanthocyanidins, saponins, sterols and triterpenes                      | Antibacterial, antiproliferation, antiprotease, antioxidant and cytotoxic                                                                                 | [121]        |
| <i>Crotalaria laburnifolia</i>     | Alkaloids                                                                                                                            | Analgesic, anthelmintic and antimicrobial                                                                                                                 | [122]        |
| <i>Dalbergia melanoxylon</i>       | Alkaloids, flavonoids, glycosides and tannins                                                                                        | Analgesic, anti-inflammatory, antimicrobial, antiviral, antioxidant and antipyretic                                                                       | [123–125]    |
| <i>Dalbergia nitidula</i>          | Flavonoids                                                                                                                           | Antibacterial, antioxidant and cytotoxic                                                                                                                  | [126,127]    |
| <i>Dalbergiella nyasae</i>         | Alkaloids, flavonoids, saponins and terpenoids                                                                                       | Antifungal and antibacterial                                                                                                                              | [128]        |
| <i>Dichrostachys cinerea</i>       | Flavonoids, phenolics, sterols, tannins and triterpenes                                                                              | Analgesic, antibacterial, anti-fungal, antiviral, anticonvulsant, anti-inflammatory, antimalarial, antioxidant, hepatoprotective and neuropharmacological | [129–132]    |
| <i>Dolichos kilimandscharicus</i>  | Flavonoids and saponins                                                                                                              | Antibacterial, anticancer, antiproliferative and cytotoxic                                                                                                | [133,134]    |
| <i>Elephantorrhiza burkei</i>      | Alkaloids, flavonoids, glycosides, phenolics, saponins, tannins and triterpenoids                                                    | Antibacterial, antifungal, anti-HIV, antidiabetic, anti-inflammatory, antioxidant, cytotoxic and mutagenic                                                | [135,136]    |
| <i>Elephantorrhiza elephantina</i> | Anthocyanidins, anthraquinones, esters, fatty acids, flavonoids, glycosides, phenolics, saponins, sterols, tannins and triterpenoids | Anthelmintic, antibacterial, antifungal, anti-inflammatory, antinociceptive, antiparasmodial and antioxidant                                              | [137]        |
| <i>Elephantorrhiza goetzei</i>     | Coumarins, flavonoids, phenolic, saponins, stilbenoids, tannins and triterpenoids                                                    | Anthelmintic, antibacterial, antifungal, antiviral, antioxidant and cytotoxic                                                                             | [138]        |
| <i>Eriosema englerianum</i>        | Volatile oils                                                                                                                        | Antibacterial and antifungal                                                                                                                              | [139]        |
| <i>Erythrina abyssinica</i>        | Alkaloids, flavonoids and terpenoids                                                                                                 | Antibacterial, antifungal, antiviral, antidiabetic, anti-inflammatory, antioxidant, antiparasmodial, antiproliferative and hepatoprotective               | [140–144]    |
| <i>Erythrina livingstoniana</i>    | Flavonoids                                                                                                                           | Antibacterial and antioxidant                                                                                                                             | [145–147]    |

Table 3. Cont.

| Species                          | Phytochemistry                                                                                                        | Pharmacological Activities                                                                                                                         | References             |
|----------------------------------|-----------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| <i>Erythrophleum africanum</i>   | Alkaloids, flavonoids, glycosides, saponins, steroids, tannins and terpenoids                                         | Antibacterial, antifungal, antidote, antioxidant and toxic                                                                                         | [148]                  |
| <i>Erythrophleum suaveolens</i>  | Alkaloids, flavonoids, sterols, stilbenoids and terpenoids                                                            | Antibacterial, antifungal, anticancer, anti-inflammatory and antioxidant                                                                           | [149]                  |
| <i>Flemingia grahamiana</i>      | Alkaloids, flavonoids, glycosides, phenolics, saponins, steroids, tannins and volatile oils                           | Antibacterial and anticancer                                                                                                                       | [150,151]              |
| <i>Indigofera arrecta</i>        | Alkaloids, flavonoids, glycosides, phenols, saponins, tannins and terpenoids                                          | Antibacterial, antiviral and anticancer                                                                                                            | [152,153]              |
| <i>Indigofera astragalina</i>    | Saponins and tannins                                                                                                  | Antioxidant and cytotoxic                                                                                                                          | [154]                  |
| <i>Indigofera hirsuta</i>        | Alkaloids, flavonoids and phenolics                                                                                   | Antidiabetic, anti-inflammatory and antioxidant                                                                                                    | [153]                  |
| <i>Indigofera spicata</i>        | Benzofuran, fatty acids, flavonoids, phthalate, rotenoids, saponins, steroids and triterpenes                         | Anticancer, antidiabetic, antidiarrhoeal, antiparasmodial and cytotoxicity                                                                         | [153,155]              |
| <i>Julbernardia globiflora</i>   | Fatty acids, lignin, proanthocyanidins and tannins                                                                    | Anticancer                                                                                                                                         | [112,156]              |
| <i>Lessertia frutescens</i>      | Amino acids, flavonoids, pinitol and triterpenes                                                                      | Analgesic, antibacterial, anticonvulsant, antidiabetic, anti-HIV, anti-inflammatory, antiproliferative, antistress and antithrombotic              | [157]                  |
| <i>Leucaena leucocephala</i>     | Coumarins, flavonoids, phytol, sterols and triterpenes                                                                | Antimicrobial, diuretic, antiviral, cytotoxic, antioxidant and anti-inflammatory                                                                   | [158–161]              |
| <i>Mucuna coriacea</i>           | None found                                                                                                            | Bacterial and antiviral                                                                                                                            | [162,163]              |
| <i>Mundulea sericea</i>          | Coumarins, flavonoids, phenolic, saponins, steroids, tannins and volatile oils                                        | Analgesic, antibacterial, antifungal, antioxidant and insecticidal                                                                                 | [164,165]              |
| <i>Neorautanenia mitis</i>       | Alkaloids, flavonoids, glycosides, saponins and tannins                                                               | Antibacterial, antifungal and antinociceptive                                                                                                      | [166,167]              |
| <i>Ormocarpum kirkii</i>         | Coumarins, flavonoids and triterpenoids                                                                               | Antibacterial, antifungal, antimalarial, antiparasmodial and cytotoxicity                                                                          | [168]                  |
| <i>Ormocarpum trichocarpum</i>   | Aliphatic hydrocarbons, coumarins, diterpenoids, steroids and triterpenes                                             | Antimicrobial, antiparasmodial, antioxidant and antimutagenic                                                                                      | [169–171]              |
| <i>Peltophorum africanum</i>     | Benzenoids, coumarins, flavonoids, glycosides, phenolics, steroids, tannins and terpenes                              | Anthelmintic, antibacterial, antifungal, antiviral, anti-inflammatory and antioxidant                                                              | [141,162,163, 172–174] |
| <i>Pericopsis angolensis</i>     | Flavonoids, saponins and tannins                                                                                      | Antimicrobial                                                                                                                                      | [107]                  |
| <i>Phaseolus vulgaris</i>        | Alkaloids, anthocyanins, esters, flavonoids, iridoids, lignans, phenolics, saponins, steroids, tannins and terpenoids | Analgesic, antibacterial, antidiabetic, anti-inflammatory, antioxidant and hypocholesterolaemic                                                    | [175,176]              |
| <i>Philenoptera violacea</i>     | Alkaloids, flavonoids, glycosides, steroids, tannins and terpenoids                                                   | Anticancer and antioxidant                                                                                                                         | [177]                  |
| <i>Piliostigma thonningii</i>    | Alkaloids, flavonoids, saponins, tannins, terpenes and volatile oils                                                  | Analgesic, anthelmintic, antibacterial, antiviral, antimalarial, anti-inflammatory, antileishmanial, antioxidant, antipyretic and immunomodulatory | [178–182]              |
| <i>Pseudarthria hookeri</i>      | Flavonoids                                                                                                            | Antibacterial and anticancer                                                                                                                       | [183]                  |
| <i>Pterocarpus angolensis</i>    | Chalcones, deoxybenzoin, fatty acids, phenolics and terpenoids                                                        | Antibacterial, antifungal, antiviral, anticancer, anti-inflammatory, antioxidant and wound healing                                                 | [184–188]              |
| <i>Pterocarpus rotundifolius</i> | Fatty acids                                                                                                           | Antiacne, antityrosinase, antioxidant and cytotoxic                                                                                                | [189]                  |
| <i>Pterolobium stellatum</i>     | Saponins, tannins and terpenoids                                                                                      | Antibacterial and antimycobacterial                                                                                                                | [190,191]              |
| <i>Rhynchosia insignis</i>       | Flavonoids                                                                                                            | Antibacterial and antifungal                                                                                                                       | [192–194]              |
| <i>Rhynchosia minima</i>         | Coumarins, flavonoids, steroids, tannins, triterpenes and volatile oils                                               | Antibacterial, antifungal and antioxidant                                                                                                          | [47,192,194]           |

Table 3. Cont.

| Species                       | Phytochemistry                                                                                                | Pharmacological Activities                                                                                                                                                  | References    |
|-------------------------------|---------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| <i>Rhynchosia resinosa</i>    | Saponins, steroids and terpenoids                                                                             | Antibacterial, antileishmanial, cytoprotective and cytotoxic                                                                                                                | [195,196]     |
| <i>Schotia brachypetala</i>   | Anthocyanins, flavonoids, glycosides, phenols and tannins                                                     | Anti-acne, antibacterial, antimalarial, antioxidant, antityrosinase and cytotoxic                                                                                           | [189,197,198] |
| <i>Senegalia ataxacantha</i>  | Alkaloids, coumarins, flavonoids, lignan, phenols, quinone, saponins, steroids, tannins and triterpenoids     | Antibacterial, antifungal, antidiabetic, anti-inflammatory, antioxidant, laxative and ulceroprotective                                                                      | [199]         |
| <i>Senegalia mellifera</i>    | Flavonoids, glycosides, phenols, saponins, tannins and terpenoids                                             | Antibacterial, antifungal and cytotoxicity                                                                                                                                  | [200–202]     |
| <i>Senegalia nigrescens</i>   | Flavonoids and triterpenoids                                                                                  | Antimicrobial, antioxidant and cytotoxicity                                                                                                                                 | [203]         |
| <i>Senna didymobotrya</i>     | Alkaloids, flavonoids, phenolics, quinones, saponins, steroids, tannins and terpenoids                        | Antibacterial                                                                                                                                                               | [204,205]     |
| <i>Senna italica</i>          | Alkaloids, anthocyanins, flavonoids, steroids and tannins                                                     | Antibacterial, antifungal, anticancer and antioxidant                                                                                                                       | [206–208]     |
| <i>Senna occidentalis</i>     | Alkaloids, anthraquinones, anthrones, flavonoids, saponins, sterols and volatile oils                         | Antibacterial, antifungal, anticancer, antidiabetic, anti-inflammatory, antimutagenic, antiprotozoal and hepatoprotective                                                   | [208,209]     |
| <i>Senna petersiana</i>       | Flavonoids                                                                                                    | Antibacterial and cytotoxic                                                                                                                                                 | [135]         |
| <i>Senna singueana</i>        | Alkaloids, anthraquinones, proanthocyanidins, phenols, saponins, sterols, tannins, terpenes and volatile oils | Antimalarial, antinociceptive, antioxidant, hepatoprotective and trypanocidal                                                                                               | [198,208]     |
| <i>Senna septemtrionalis</i>  | Anthraquinones, benzoic acids, carboxylic acids and flavonoids                                                | Anticonvulsant, anti-inflammatory, diuretic and antinociceptive                                                                                                             | [208,210,211] |
| <i>Sphenostylis erecta</i>    | Flavonoids and sphenostylins                                                                                  | Antifungal, antioxidant and cytotoxicity                                                                                                                                    | [212]         |
| <i>Tamarindus indica</i>      | Amino acids, fatty acids and tannins                                                                          | Antibacterial, antifungal, antiviral, antidiabetic, anti-inflammatory, antinematodal, antioxidant, cytotoxic and molluscicidal                                              | [213]         |
| <i>Tylosema fassoglense</i>   | Alkaloids, flavonoids, glycosides, phenolics, quinones, saponins, steroids, tannins and terpenoids            | Antibacterial and anticancer                                                                                                                                                | [214,215]     |
| <i>Vachellia karroo</i>       | Flavonoids, phenols, proanthocyanidin, sterols, tannins and terpenoids                                        | Analgesic, antibacterial, antifungal, antiviral, antihelmintic, anti-inflammatory, antimalarial and antioxidant                                                             | [216]         |
| <i>Vachellia nilotica</i>     | Alkaloids, fatty acids, flavonoids and tannins                                                                | Inhibition of acetylcholinesterase, anthelmintic, antibacterial, anticancer, antihypertensive, anti-inflammatory, antioxidant and antiplatelet                              | [217]         |
| <i>Vachellia rehmanniana</i>  | None found                                                                                                    | Anti-inflammatory                                                                                                                                                           | [218]         |
| <i>Vachellia sieberiana</i>   | Flavonoids, glycosides, phenolics, quinones, saponins and tannins                                             | Antibacterial and anticancer                                                                                                                                                | [219,220]     |
| <i>Vigna unguiculata</i>      | Flavonoids and phenolics                                                                                      | Acetylcholinesterase inhibition, anthelmintic, antibacterial, antifungal, antiviral, antidiabetic, anti-inflammatory, antioxidant, antinociceptive and hypocholesterolaemic | [221–225]     |
| <i>Xeroderris stuhlmannii</i> | Alkaloids, flavonoids, phenols, steroids and terpenoids                                                       | Antibacterial, antiviral, anticancer, anti-inflammatory, antioxidant and antiproliferative                                                                                  | [226–228]     |

\* “No report found” means that no record of the phytochemical or pharmacological properties were found in the literature.

#### 4. Conclusions

This review is a compilation of literature sources on the Fabaceae species used as traditional medicines in Zimbabwe, providing an important repository of ethnopharmacological data required for future studies. The Fabaceae family is characterized by several species used as traditional medicines for the treatment and management of different ailments and diseases. The literature search showed that there is a paucity of information on the cultural practices associated with usage of Fabaceae species, including information on their dosages and administration. Therefore, there is a need for ethnobotanical research into and documentation of the cultural value of the Fabaceae species in Zimbabwe. Fabaceae species that are exotic to Zimbabwe are also used as sources of traditional medicines, corroborating the general observation that traditional pharmacopoeias are not static social institutions but fluid and dynamic, characterized by the addition of exotic plant species as herbal medicines.

Several Fabaceae species used as traditional medicines are known to contain bioactive compounds which have demonstrated diverse pharmacological properties against several disease-causing pathogens. Plant extracts and phytochemical compounds isolated from Fabaceae species have shown inhibition of the acetylcholinesterase enzyme and many other properties, such as antitumor, antidiabetic, antifertility, anthelmintic, antiamebic, anti-inflammatory, antimicrobial, antioxidant, antiparasitic, cytotoxic, hepatoprotective, hypoglycaemic and immunomodulatory. However, the majority of the studied biological activities have mainly been in vitro assays, while clinical and in vivo studies are lacking. It is recommended that the unstudied biological activities of the medicinal species should be investigated to unravel the therapeutic potential of the considered Fabaceae species, using both in vitro and in vivo models. Furthermore, the toxicological properties of these species should be evaluated and the mechanism of action of the identified phytochemicals should be elucidated based on their pharmacological properties.

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## Article

# Analysis of the Toxicological Profile of *Heracleum sosnowskyi* Manden. Metabolites Using *In Silico* Methods

Anna E. Rassabina \* and Maxim V. Fedorov \*

Institute for Information Transmission Problems of the Russian Academy of Sciences (Kharkevich Institute),  
Bolshoy Karetny per. 19, Moscow 127051, Russia

\* Correspondence: rassabina.ae@iitp.ru (A.E.R.); fedorov@iitp.ru (M.V.F.)

**Abstract:** The invasive plant *Heracleum sosnowskyi* Manden. is a valuable source of a number of bioactive metabolites that can be used in the pharmaceutical industry and medicine and may have some other applications as well. Today, there is a need to summarize data on these substances as well as analyze the toxicological profile of the metabolites of *H. sosnowskyi*. In this study, we collected a dataset of 225 metabolites of *H. sosnowskyi* from different literature sources and performed cluster analysis of their chemical structures; we revealed five main clusters of compounds: terpenoids, aromatic compounds, polyaromatic compounds, fatty acids, and furanocoumarins. In order to fill the gaps in the experimental data on the toxicity of the studied substances, we used machine learning (ML) algorithms previously designed for high-accuracy prediction of toxicity end-points. The ML-based approach allowed us to fill in up to 90% of the missing median lethal dose  $LD_{50}$  (mouse) data for the studied molecules. The validity of each predicted value was confirmed by analyzing the applicability domain of the used ML models. For the calculations and ML modeling, we used the *Syntelly* chemoinformatics platform. For the most toxic compounds—hydroxycoumarins and furanocoumarins of *H. sosnowskyi*—the values for hepatotoxicity, drug-induced liver injury (DILI), cardiotoxicity, and carcinogenicity were predicted. Based on the analysis of  $LD_{50}$  values for the mouse animal model, the greatest toxicity for furanocoumarins is expected with the intravenous route of administration (62–450 mg/kg), which can cause drug-induced liver injury. At the same time, the data do not show high cardiotoxicity risks for the studied furanocoumarins. Based on the presented results, we discuss prospects of using some of the compounds as pharmaceutical agents.

**Keywords:** chemoinformatics; furanocoumarins; *Heracleum sosnowskyi* Manden.; metabolites; toxicity

## 1. Introduction

Today, special attention is being paid to invasive plants that actively reproduce, disperse, and integrate into natural plant communities outside their natural range, causing significant environmental and economic damage to native species and ecosystems [1,2]. Among the numerous invasive species, there is *Heracleum sosnowskyi* Manden. (*Apiaceae*) (Sosnowski's hogweed) (*H. sosnowskyi*) [3]. It is known that *H. sosnowskyi* has spread widely throughout several countries in Eastern and Northern Europe, as well as in the Baltic States, after it was introduced as a forage plant [4–7].

Due to their diverse chemical composition, invasive plants are able to easily adapt to various environmental conditions and successfully compete with native species. It

is known that secondary metabolites of plants are important sources for the discovery of new medicines [8]. Secondary metabolites are usually multifunctional compounds capable of exhibiting various pharmacological properties. Along with many members of the *Apiaceae* family, *H. sosnowskyi* contains metabolites that exhibit photosensitizing, antioxidant, cytotoxic, antimicrobial, and antitumor activities [9]. Carbohydrates, proteins, aldehydes, esters, and phenolic compounds have been shown to occur in *H. sosnowskyi* [10–12]. Indeed, pectin substances and proteins were found in aqueous extracts of *H. sosnowskyi*, while alkaloids, carotene, and furanocoumarins were found in alcoholic extracts [13]. For example, 38 fatty acids have been identified in extracts obtained from various parts of *H. sosnowskyi* biomass [14]. In addition to hemicellulose polysaccharides [15], the biomass of *H. sosnowskyi* also contains glutamine, vitamins C and P, folic acid, 17 amino acids, as well as various macro- and microelements [16]. The leaves, stems, seeds, and inflorescences of *H. sosnowskyi* are of the greatest medicinal value, as they serve as sources of the most biologically active compounds [17]. This species can be considered a promising source of natural bioactive substances [18,19].

The most discussed compounds extracted from *H. sosnowskyi* are furanocoumarins, which have pronounced photosensitizing activity [20,21]. A significant diversity of furanocoumarins characterizes the phytochemical profile of *H. sosnowskyi* extract, among which are bergapten, angelicin, psoralen, xanthotoxin, pimpinellin, isopimpinellin, and sphondin [22]. The quantitative content of some furanocoumarins in a plant ranges from 0.15 mg/g to 3.14 mg/g of plant material [18]. When exposed to ultraviolet (UV) radiation, the activity of furanocoumarins increases, and, when they come into contact with human skin, they can cause severe burns [19,23].

There are linear furanocoumarins, which are derivatives of psoralen, and angular derivatives of angelicin [24,25]. It should be noted that, according to the literature, up to 400 different furanocoumarins with biological activity can be found in plants of the *Apiaceae* family alone [26].

Many metabolites of *H. sosnowskyi* are toxic to humans and animals [27]. Various toxicity end-points of some of the compounds have been studied by various animal models *in vitro* [19,28]. The toxicological properties of *H. sosnowskyi* metabolites are mainly associated with chemical classes such as alkaloids, triterpene saponins, flavonoids, and furanocoumarins [22].

Overall, there are a number of reports showing numerous toxic effects of the juice and different parts of the plant on humans and farm animals [17,29]. However, data on the toxicity end-points of the metabolites of *H. sosnowskyi* appear scattered. Therefore, it is necessary to summarize and analyze the available experimental data within the framework of an integrated approach and fill in the missing information.

There are a wide range of experimental methods for assessing toxicity, including *in vitro* and *in vivo*, but they require a large amount of time and financial resources. Therefore, due to the development of machine learning (ML) [30], the approach of predicting various toxicity parameters using *in silico* methods is currently becoming increasingly popular. Computational toxicology based on ML for predicting the toxicity of metabolites helps fill gaps in experimental data and perform an express assessment of toxicity for new compounds [31]. Chemoinformatics and ML methods make it possible to analyze large amounts of data as well as predict properties even for poorly studied compounds [32]. The quantitative structure–activity relationship (QSAR) model is widely used to predict the toxicity of compounds based on their structure [23,33]. Thus, the modular artificial intelligence platform *Syntelly* [34] allows one to analyze the physico-chemical properties as well as biological and toxicological activity of many chemical compounds [35,36].

The purpose of our study is to summarize data, cluster molecular structures, and predict the median lethal dose  $LD_{50}$  (mouse) of metabolites of *H. sosnowskyi* *in silico*, as well as to establish hepatotoxicity, drug-induced liver injury (DILI), cardiotoxicity, and carcinogenicity of furanocoumarins from *H. sosnowskyi*. Based on these data, we discuss the risks associated with these compounds. In addition, we estimate the cost of synthesis of several selected compounds to assess their perspectives on the market.

## 2. Materials and Methods

### 2.1. Data Preparation

Data on the presence of these compounds in *Heracleum sosnowskyi* Manden. were obtained by analyzing publications contained in the PubMed, PubChem, and SciFinder databases and peer-reviewed articles with the keywords “furanocoumarins of *Heracleum sosnowskyi*”, “chemical composition of *Heracleum sosnowskyi*”, etc. The presence of compounds was previously experimentally confirmed and taken into account by us, regardless of their established amount in raw plant materials of *H. sosnowskyi*.

### 2.2. Establishing Canonical SMILES

For each substance, a search was performed for the CAS number and Canonical SMILES in the PubChem database using the Python 3.12 RDKit package. In the absence of a corresponding CAS number and canonical SMILES for the compounds [37], a structure search was performed on the Russian modular artificial intelligence platform *Syntelly* [34].

### 2.3. Clusterization of Data

Clustering was performed using the SynMap module of the *Syntelly* platform. The method of studying chemical space is based on the parametric multiscale t-SNE method in combination with differential fingerprints [38].

### 2.4. In Silico Toxicity Analysis

Aggregation and prediction of the median lethal dose  $LD_{50}$  (mouse) (mg/kg) were performed on the *Syntelly* platform for mouse test objects for various routes of administration: oral, intravenous, intraperitoneal, mouse skin, subcutaneous, and intramuscular. Experimental data were collected from TOXRIC (<https://toxic.bioinformai.tech>, accessed on 1 July 2025), ChemIDplus, NIH (<https://www.nih.gov>, accessed on 1 July 2025), NCATS-Flux, PyTDC, and PubMed platforms. Analysis was performed of hepatotoxicity, drug-induced liver injury (DILI), cardiotoxicity, and carcinogenicity on the *Syntelly* platform in the Datasets module using models. CatBoost and XGBoost algorithms were used [36]. The performance of the fingerprint-based CatBoost and fragment-based XGBoost algorithms was evaluated using RMSE metric for regression tasks and ROC-AUC for classification tasks [36].

### 2.5. Assessment of the Applicability Domain

For each predicted toxicity value, the applicability domain (AD) was evaluated. AD was determined based on the structural similarity between the test molecules and the training set [39]. The molecules were projected into a 2D chemical space (SynMap module), after which the average distance to the nearest neighbors was calculated using the kNN ( $k = 5$ ) method. The obtained value was normalized relative to a predefined threshold distance for each model and transformed using a Gaussian function. The final indicator of AD (0–1) reflects the reliability of the prediction: values  $\geq 0.5$  indicate the reliability of the prediction, values  $< 0.5$ —beyond the limits of AD. Then they are represented as



percentages (*Syntelly*), where 0–20% is low reliability of prediction, 20–50% is average reliability of prediction, and 50–100% is high reliability of prediction.

### 2.6. Cost of Synthesis

The cost of synthesis was estimated by the *Syntelly* platform using the Synthesis cost module. The search was carried out using SMILES of the corresponding compound based on 1 g, and the number of stages per synthesis ranged from 1 to 6 [34].

### 2.7. Statistical Analysis

The JASP version 0.19.3 program was used for statistical data analysis. The normal distribution was analyzed using the graphical method and the Shapiro–Wilk criterion. The median toxicity was calculated as well as the 95% confidence interval for the median. The results in Supplementary Table S6 are presented as average values with standard deviations ( $\pm$ SDs) obtained after a five-fold check ( $n = 5$ ).

## 3. Results

### 3.1. Dataset on *Heracleum sosnowskyi* Metabolites

Data on *Heracleum sosnowskyi* Manden. metabolites were collected from literature sources and peer-reviewed articles for the period 2003–2025 from the PubChem, SciFinder, and PubMed databases. The dataset includes compounds (as well as their known isomers) whose presence in *H. sosnowskyi* has been experimentally confirmed based on the literature. We compiled a dataset of 225 metabolites identified to date in *H. sosnowskyi*. According to the literature, compounds such as pabulenol, columbianetin, heraclenin, alloisoimperatorin, and heraclesol have been found in *H. sosnowskyi* in various parts of the plant [40]. Bergamot-tin plays a key role in the cytotoxic activity of bergamot essential oil [21] and demonstrates protumor activity [25]. The toxicity of phellopterin, scopoletin, and umbelliferone was observed, along with the toxicity of the aqueous extract of *H. sosnowskyi* [28].

For analysis, we used compounds whose presence in the plant has been experimentally proven. Therefore, some of the compounds mentioned in the literature as potential components of *H. sosnowskyi* but without a clear indication of their actual presence in the plant were not included in the dataset to avoid confusion. Table 1 presents some major compounds found in *Heracleum sosnowskyi*, including coumarins.

**Table 1.** Content of chemical substances in different parts of *Heracleum sosnowskyi*.

| Parts of the Plant | Substance        | Content, mg/g * | References |
|--------------------|------------------|-----------------|------------|
| Leaves             | Triterpenes      | 7.4             | [14]       |
|                    | Terpenes         | 0.6             | [14]       |
|                    | Furanocoumarins  | >6.4            | [18]       |
| Seeds              | Carboxylic acids | 21.0            | [14]       |
|                    | Coumarins        | 22.8            | [14]       |
| Stems              | Carboxylic acids | 1.1             | [14]       |
|                    | Terpenes         | <0.1            | [14]       |
|                    | Alcohols         | <0.1            | [14]       |

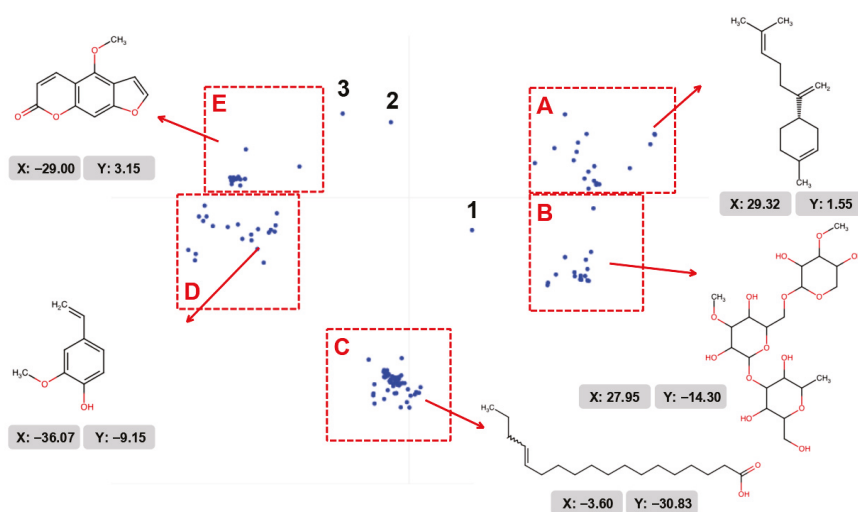
\* Dry weight of raw materials (DW).

### 3.2. Clustering of Metabolites

The clustering method, which is widely used to evaluate the chemical space of many molecules, was also used in our study, where clusters of metabolites were formed based on their molecular similarity. Clustering was performed by the *Syntelly* platform using

the SynMap module after uploading a set of molecular data to the platform in the form of standardized SMILES (Supplementary Tables S1–S5).

It was found that 225 metabolites are distributed in five main clusters (Figure 1). In Cluster A, 25 compounds are mainly represented by terpenoids (isoprenoids) containing cyclohexane, bicyclic, or polycyclic fragments. Cluster B contains 22 compounds, such as polyphenolic glycosides, flavonoids, and cyclic polyols. The populous cluster C is represented by 132 fatty acids. Aromatic molecules with phenyl rings and derivatives of phenolic compounds are represented in cluster D, with a total of 21 compounds. Cluster E includes 22 compounds, mainly furanocoumarins. Cluster E also includes quininic acid and coumarin derivatives. It was found that three molecules were not included in the clusters: molecule 1 was (4Z)-1-methyl-4-(6-methylhept-5-en-2-ylidene)cyclohexene ( $\gamma$ -bisabolene, (z)-) a non-aromatic terpene without functional groups (–OH, –COOH) with a specific stereochemistry (Z-configuration) that was not included in cluster A. The compound 1-methyl-4-propan-2-ylidenecyclohexene ( $\alpha$ -terpinolene) (molecule 2) is a non-aromatic terpene, which is why it did not fall into the terpenoid cluster, but, if cluster A was expanded to terpenes and their derivatives, it could well be included. And, 9-[(3,3-dimethyloxiran-2-yl)methoxy]-4-methoxyfuro[3,2-g]chromen-7-one (byakangelicol)—a furanocoumarin with an epoxy group—and the count of the epoxy group (3,3-dimethyloxiran) may differ from the furanocoumarin cluster (molecule 3) (Figure 1). We will consider the three connections that are not included in the clusters separately.



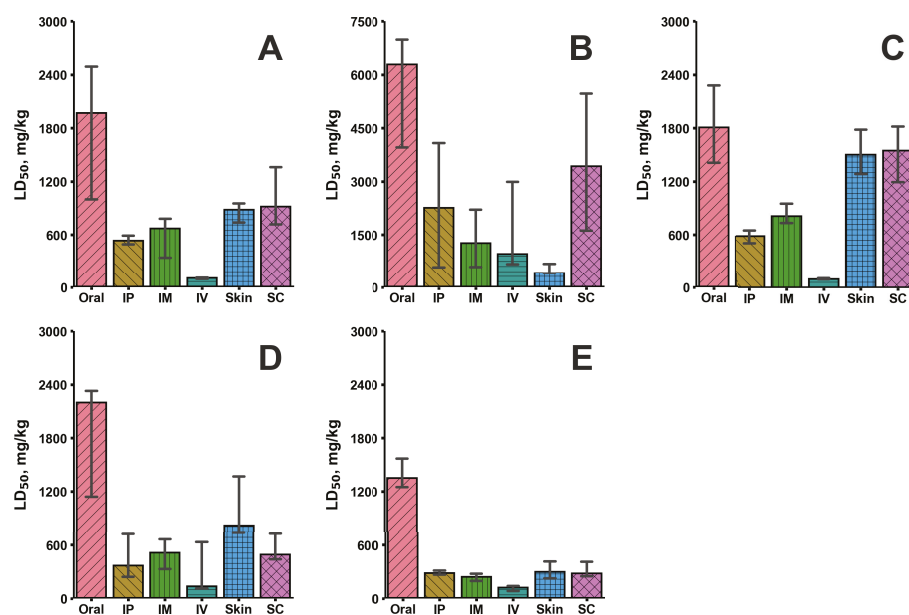
**Figure 1.** Two-dimensional clustering map of the metabolites found in *H. sosnowskyi* using the SynMap module of the Syntelly platform. The construction of clusters is based on the structural similarity of molecules. Clusters of molecules: A—terpenoids (isoprenoids), B—polyphenolic glycosides, flavonoids, and cyclic polyols, C—fatty acids, D—aromatic compounds, E—furanocoumarins and quinolines. The callout shows examples of connections presented in this cluster with coordinates in two-dimensional space. Connections that are not included in clusters: 1— $\gamma$ -bisabolene, (z)-; 2— $\alpha$ -terpinolene; 3—byakangelicol.

### 3.3. In Silico Toxicity Prediction and Applicability Domain Assessment

For five clusters of compounds, the prediction of  $LD_{50}$  (mouse) *in silico* was performed on test subjects in mice with various routes of administration: oral, intravenous, intraperitoneal, mouse skin, subcutaneous, and intramuscular on the Syntelly platform (Figure 2). In addition to the prognostic data of toxicity *in silico*, the platform aggregates experimental  $LD_{50}$  (mouse) (Exp) data on some compounds.

Figure 2 shows the median toxicity values of each cluster of compounds for different routes of administration. Note that the values of the RMSE metric for regression prediction models for oral, intravenous, and intraperitoneal routes of administration averaged 45%, which indicates acceptable accuracy (Supplementary Table S6).

In a comprehensive analysis of the toxicity of compounds, we consider oral and intravenous routes of administration as the most preferred routes of drug administration. We have shown that among the five clusters of compounds in *H. sosnowskyi*, cluster E has the highest median toxicity by oral administration in mice, with  $LD_{50}$  (mouse) values in the range of 423–8100 mg/kg. A cluster of fatty acids showed significant toxicity, followed by a cluster of terpenoids, followed by a cluster of aromatic compounds and polyphenolic glycosides. It is worth noting that the toxicity of the compounds depends on their route of administration, and the highest toxicity of cluster E was observed with the intravenous route of administration (62–450 mg/kg) (Figure 2). For molecules not included in the cluster, it was noted that byakangelicol has an  $LD_{50}$  (mouse) of 94 mg/kg with the intravenous route of administration, for  $\gamma$ -bisabolene ((z)-), 401 mg/kg, and for  $\alpha$ -terpinolene, 133 mg/kg. With the oral route of administration, these compounds did not show pronounced toxicity (over 1000 mg/kg).



**Figure 2.** Median toxicity  $LD_{50}$  (mouse) values, mg/kg for different routes of administration: Oral—oral, IP—intraperitoneal, IM—intramuscular, IV—intravenous, Skin—mouse skin, and SC—subcutaneous. Five clusters of metabolites are presented for *H. sosnowskyi*, ranking compounds by predicted toxicity, where (A)—terpenoids (isoprenoids), (B)—polyphenolic glycosides, flavonoids, and cyclic polyols, (C)—fatty acids, (D)—aromatic compounds, and (E)—furanocoumarins. The vertical line specifies a 95 percent confidence interval of the median.

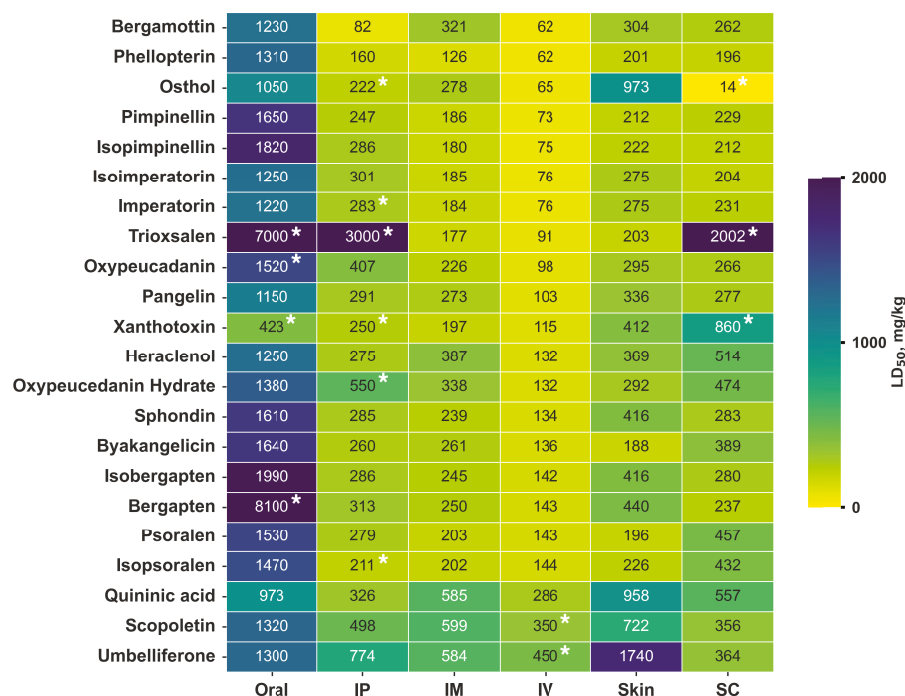
For the predicted  $LD_{50}$  (mouse) values, the applicability domain (AD) of the QSAR model was calculated, which defines the boundaries within which the model's predictions are considered reliable [41] (Supplementary Table S6). If the values are in the range from 0 to 20%, then there are few molecules similar to the selected one in the training data of the model, which corresponds to low prediction reliability. The range of 20–50% corresponds to average reliability of prediction, and the range of 50–100% corresponds to high reliability. Of the five clusters of compounds in *H. sosnowskyi*, the highest AD values were observed in cluster D for aromatic compounds. It can be assumed that there are probably more aromatic compounds in the training sample of experimental data, and the model predicted

the values better (Supplementary Figure S1). Cluster E comes in second place, followed by cluster B with polyphenolic compounds and flavonoids, followed by cluster A with terpenoids and cluster C with fatty acids.

### 3.4. Analysis of *H. sosnowskyi* Metabolite Toxicity

For cluster E, which contains the most toxic compounds from *H. sosnowskyi*, a toxicity spectrum heatmap was constructed, where the brightest fragments correspond to the most toxic compounds (Figure 3). Recall that furanocoumarins and coumarin derivatives are the least toxic with the oral route of administration, while they exhibit the greatest toxicity with intravenous administration. The most toxic furanocoumarin is bergamottin; its intravenous  $LD_{50}$  (mouse) is 62 mg/kg (AD 74%) and that of phellopterin for intravenous route of administration is 62 mg/kg (AD 60%). The least toxic are scopoletin and umbelliferone, for which the  $LD_{50}$  (mouse) of an intravenous application is 350 and 450 mg/kg, respectively. When comparing the values presented by the *Syntelly* platform with the data from the articles [28], the experimental data for xanthotoxin (423 mg/kg) for the  $LD_{50}$  (mouse) of furanocoumarins coincide.

For compounds of the E cluster of *H. sosnowskyi*, we were able to predict the majority of toxicity values for different routes of administration, and it is worth noting that for regression models, AD for oral, intravenous, and intraperitoneal routes of administration exceeded 50% confidence (Supplementary Figure S2). For intramuscular and mouse skin, the values of the AD did not exceed 40% on average. This was probably due to the peculiarities of the data on which the model was trained. Of all the toxicity values that were selected for complex analysis, only 10% of the total number were experimental (Figure 3), which indicates a high demand for filling in the missing data.



**Figure 3.** Heatmap of the toxicity spectrum  $LD_{50}$  (mouse), mg/kg, of cluster E furanocoumarins found in *H. sosnowskyi*, where Oral—oral, IP—intraperitoneal, IM—intramuscular, IV—intravenous, Skin—mouse skin, and SC—subcutaneous. Both the predicted *in silico* values and the experimental values (\*) are presented. The ranking is based on the intravenous (IV) route of administration. The color indicates the toxicity of the molecules: dark purple indicates low toxicity, and yellow indicates high toxicity.

### 3.5. Prediction of General Toxicity In Silico

In addition to analyzing the toxicity of  $LD_{50}$  (mouse), we aggregated experimental data (Exp) and obtained prognostic data *in silico* on hepatotoxicity, drug-induced liver injury (DILI), cardiotoxicity, and carcinogenicity of cluster E (Table 2). For the binary classification of toxic/nontoxic, the values of the ROC-AUC ranged from 79% to 93% (Supplementary Table S6), which shows the high efficiency of prediction models. It is shown that furanocoumarins from *H. sosnowskyi* can cause drug-induced liver injury, while they have a low risk of cardiotoxicity (Table 2). As a result, it is predicted that 5 out of 22 compounds do not have hepatotoxicity. It is worth noting that the AD of the hepatotoxicity parameter for many values was higher than 60%, with the exception of 4-[(3,3-dimethyloxiran-2-yl)methoxy]furo[3,2-g]chromen-7-one ((R)-Oxypeucedanin) and 6-methoxyquinoline-4-carboxylic acid (quininic acid), for which the AD of hepatotoxicity was 38% and 29%, respectively. We found an absence of carcinogenicity for five compounds in cluster E, which had an AD over 45%.

**Table 2.** Data on hepatotoxicity, drug-induced liver injury (DILI), cardiotoxicity, and carcinogenicity of cluster E compounds isolated from *Heracleum sosnowskyi* and applicability domain (AD) values in % for each predicted value.

| Substance                          | Hepatotoxicity |          | DILI   |      | Cardiotoxicity |            | Carcinogenicity |       |
|------------------------------------|----------------|----------|--------|------|----------------|------------|-----------------|-------|
|                                    | Effect         | AD,%     | Effect | AD,% | Effect         | AD,%       | Effect          | AD,%  |
| Bergamottin <sup>a</sup>           | Toxic          | 90       | Toxic  | 41   | Nontoxic       | 67         | Nontoxic        | 48    |
| Phellopterin <sup>a</sup>          | Toxic          | 91       | Toxic  | 41   | Nontoxic       | PyTDC      | Nontoxic        | 58    |
| Osthol <sup>b</sup>                | Toxic          | TOXRIC * | Toxic  | 48   | Nontoxic       | PyTDC      | Toxic           | 79    |
| Pimpinellin <sup>a</sup>           | Toxic          | 88       | Toxic  | 40   | Nontoxic       | 51         | Nontoxic        | 51    |
| Isopimpinellin <sup>a</sup>        | Toxic          | 79       | Toxic  | 37   | Nontoxic       | PyTDC      | Nontoxic        | 45    |
| Isoimperatorin <sup>a</sup>        | Toxic          | TOXRIC   | Toxic  | 46   | Nontoxic       | PyTDC      | Toxic           | 76    |
| Imperatorin <sup>a</sup>           | Toxic          | TOXRIC   | Toxic  | 43   | Nontoxic       | PyTDC      | Nontoxic        | 61    |
| Trioxsalen <sup>a</sup>            | Toxic          | TOXRIC   | Toxic  | 53   | Nontoxic       | NCATS-Flux | Toxic           | 88    |
| Oxypeucedanin <sup>a</sup>         | Toxic          | 38       | Toxic  | 29   | Nontoxic       | PyTDC      | Toxic           | 26    |
| Pangelin <sup>a</sup>              | Toxic          | 61       | Toxic  | 41   | Nontoxic       | PyTDC      | Nontoxic        | 77    |
| Xanthotoxin <sup>a</sup>           | Toxic          | 89       | Toxic  | 41   | Nontoxic       | PyTDC      | Toxic           | 45    |
| Heraclenol <sup>a</sup>            | Nontoxic       | 92       | Toxic  | 41   | Nontoxic       | PyTDC      | Nontoxic        | 58    |
| Oxypeucedanin Hydrate <sup>a</sup> | Nontoxic       | 90       | Toxic  | 41   | Nontoxic       | PyTDC      | Nontoxic        | 57    |
| Sphondin <sup>a</sup>              | Toxic          | 89       | Toxic  | 46   | Nontoxic       | PyTDC      | Toxic           | 61    |
| Byakangelicin <sup>a</sup>         | Nontoxic       | 89       | Toxic  | 40   | Nontoxic       | PyTDC      | Nontoxic        | 47    |
| Isobergapten <sup>a</sup>          | Toxic          | 82       | Toxic  | 42   | Nontoxic       | 54         | Toxic           | 47    |
| Bergapten <sup>a</sup>             | Toxic          | TOXRIC   | Toxic  | 41   | Nontoxic       | NCATS-Flux | Toxic           | 48    |
| Psoralen <sup>a</sup>              | Toxic          | TOXRIC   | Toxic  | 51   | Nontoxic       | PyTDC      | Toxic           | 79    |
| Isopsoralen <sup>a</sup>           | Toxic          | 93       | Toxic  | 42   | Nontoxic       | 23         | Toxic           | 54    |
| Quininic acid <sup>c</sup>         | Nontoxic       | 29       | Toxic  | 63   | Nontoxic       | 66         | Nontoxic        | 49    |
| Scopoletin <sup>b</sup>            | Nontoxic       | TOXRIC   | Toxic  | 51   | Nontoxic       | PyTDC      | Nontoxic        | PyTDC |
| Umbelliferone <sup>b</sup>         | Toxic          | TOXRIC   | Toxic  | 57   | Nontoxic       | 67         | Nontoxic        | 46    |

<sup>a</sup> Furanocoumarin; <sup>b</sup> coumarin; <sup>c</sup> cyclohexanecarboxylic acid. \* TOXIC and PyTDC are special designations used in the model.

### 3.6. Estimation of the Cost of Synthesis

Using the Synthesis cost module of the *Syntelly* platform, the synthesis cost was estimated for three representative compounds from cluster E. The highest estimated cost for the synthesis of xanthotoxin from 2-hydroxybutanedioic acid and 2,3-dihydro-1-benzofuran-6,7-diol was USD 311 per 1 g of product. The cost of psoralen synthesis turned out to be about 12.5 times lower than that of xanthotoxin. Interestingly, cost estimations correlate



with the content of these compounds in the plant material of *H. sosnowskyi*: xanthotoxin, which is more expensive to synthesize, has a higher natural content (0.8 mg/g) compared to psoralen (0.2 mg/g) [18].

The lowest estimation of the synthesis cost was found for hydroxycoumarin umbelliferone (a pathway from benzoyl chloride would cost USD 0.19 per 1 g of the product). Given its key role in the biosynthesis of coumarins and its importance in applications [42], it is of the greatest interest as a promising object for chemical synthesis, with the possibility of subsequent modification into more complex derivatives, for example, furanocoumarins. At the same time, umbelliferone does not show cardiotoxicity and carcinogenicity (Table 2), which correlates well with experimental observations of its low toxicity [43].

#### 4. Discussion

Currently, there is a need for a comprehensive analysis of the toxicological profile of *H. sosnowskyi* metabolites because of (1) risks associated with the wide and uncontrolled distribution of this invasive plant, and (2) the plant may become a promising source of biologically active substances. The toxicity data on the compounds available in the literature are incomplete and fragmented. Therefore, we believe that the presented study fills some of the gaps, and it may form a deeper understanding of the chemical composition of the invasive plant.

Due to the growing popularity of using QSAR [44], predictive models should be validated, and the most important element of this process is a clear definition of the model's applicability domain (AD) [45]. The AD is calculated based on the comparison of the predicted molecule with the molecules of the training sample for each model. Thus, the assumption here is that if the molecule is structurally similar to the training sample, then we believe that the model can make predictions for it. The molecules of the training sample and the predicted molecule are projected onto a map of the chemical space [46] (a 2D model used in the SynMap module). Then the kNN method is used—the average distance from the predicted molecule to the five nearest neighbors is calculated. For each model, the distance is defined in advance for properties, the boundary beyond which the model is considered inapplicable. The calculated average distance is normalized relative to the boundary, and then a Gaussian function is applied to this value. As a result, we obtain the values of the AD. For metabolites of a phenolic nature, toxicity values with a high AD index can be obtained. We confirmed that furanocoumarins from *H. sosnowskyi* are the most toxic molecules among the compounds of a phenolic nature. The analysis of clusters of terpenoids and fatty acids seems promising, but their detailed analysis was outside the scope of the presented work.

We collected a dataset of 225 metabolites from *H. sosnowskyi*, which contains standardized SMILES of molecules and SynID (Supplementary Tables S1–S5). The dataset contains both *in vitro* experimental data (aggregated data obtained by HPLC, GC-MS/MS) and predicted *in silico* data. However, the authors do not exclude the possibility that *H. sosnowskyi* may contain a wide range of other compounds that need to be investigated in order to form a complete picture of the toxicity of all secondary metabolites, and this dataset will be updated in the future as soon as new experimental data are found in the literature.

Our analysis revealed that phenolic compounds and lipids constituted the largest proportion of metabolites in the dataset, which can be attributed to their high abundance in *H. sosnowskyi* [14,47]. It was previously shown that leaf extracts had a high content of triterpenes (7.4 mg/g DW) (Table 1) [14], and the total furanocoumarin content in the leaves of *H. sosnowskyi* was >6.4 mg/g [18].



We reported the toxicological profile of furanocoumarins, quininic acid, and some coumarins found in *H. sosnowskyi*. At the same time, we did not take into account the synergism of compounds *in vivo*, and there are no data on chronic toxicity.

Furanocoumarins are known to be actively used as pharmaceuticals, despite the fact that they have pronounced toxicity [48,49]. To assess the potential use of raw plant materials as a source of secondary metabolites for the production of medicines, it is important to precisely identify the different types of general toxicity (hepato-, cardio-, carcinogenicity) of all furanocoumarins studied. Analysis of hepatotoxicity, cardiotoxicity, including DILI [50,51], and carcinogenicity of poorly studied furanocoumarins is necessary for assessing the risks associated with pharmaceutical preparations based on raw plant materials from *H. sosnowskyi*. The DILI parameter will allow us to evaluate the effectiveness of the use of furanocoumarins as medicines. The values of hepatotoxicity for 15 furanocoumarins were revealed (Table 2).

The calculation of the cost of hydroxycoumarin and furanocoumarin synthesis will make it possible to evaluate the possibility of obtaining compounds using chemical synthesis. We showed that the cost of these compounds varies from USD 0.19 to 311 per 1 g. The synthesis of umbelliferone is potentially cheap and can be scaled for practical applications. Thus, we showed that there is variation in the cost of synthesis of furanocoumarins and their derivatives, which must be taken into account during further extraction or synthesis.

Certainly, the data obtained on *in silico* toxicity require confirmation by *in vitro* methods, but this comprehensive study will optimize the selection of promising compounds for subsequent stages of *in vivo* testing and reduce time and financial costs by minimizing experiments. Thus, the integration of *in silico* and *in vitro* methods will provide a more complete and reliable assessment of the toxicity of metabolites, which is especially important in the development of drugs and other bioactive compounds.

## 5. Conclusions

We compiled a dataset of 225 metabolites reported in *H. sosnowskyi* to date, based on data in the literature. We provide their chemical structures and discuss their toxicological properties in detail using both available experimental data and ML-based estimations in those cases where experimental data were not available. This provides a source of data for future studies of the chemotype of the plant. The dataset also gives some background for a systematic analysis of its usage in the biotech and pharmaceutical industries. It may be used as a benchmark for future studies of the mechanisms of its profound invasive capabilities compared to other relative species.

As a result of the conducted analysis, it was established that the main toxicological hazard of *H. sosnowskyi* is associated with furanocoumarins and coumarin derivatives. The highest toxicity of the selected furanocoumarin cluster was observed for intravenous administration in  $LD_{50}$  (mouse), ranging from 62 to 450 mg/kg. The data obtained are of fundamental and practical importance, important for assessing the risks of using *H. sosnowskyi* metabolites as medicines, as well as for obtaining their synthetic analogues.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/plants14213253/s1>. Tables S1–S5: Standardized SMILES and corresponding identification numbers (SynID) for clusters A–D; Table S6: Main model quality parameters; Figure S1: Heatmap of the values of the applicability domain (AD, %) for each predicted acute  $LD_{50}$  (mouse) for cluster D of *Heracleum sosnowskyi* with various routes of administration. EXP is the experimental values aggregated from the ChemIDplus, Toxic, PyTDC, ECHA, NIH, and PubMed platforms. The value 0 indicates the inability to predict the values for these molecules; Figure S2:

Heatmap of the values of the applicability domain (AD, %) for each predicted acute  $LD_{50}$  (mouse) cluster E of *Heracleum sosnowskyi* with various routes of administration. EXP is the experimental values aggregated from the ChemIDplus, Toxic, PyTDC, ECHA, NIH, and PubMed platforms. The value 0 indicates the inability to predict the values for these molecules.

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**Data Availability Statement:** Most of the data are provided in the Supplementary Materials to this paper. Other data supporting the results of this study can be obtained from the corresponding authors upon reasonable request.

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## Article

# Antioxidant-Rich *Clitoria ternatea* Flower Extract Promotes Proliferation and Migration of Human Corneal Epithelial Cells

Karthini Devi Rajan, Nahdia Afifah Abdul Jalil, Taty Anna Kamarudin and Fairus Ahmad \*

Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM), Jalan Yaacob Latif, Bandar Tun Razak, Kuala Lumpur 56000, Malaysia; karthinidevi92@gmail.com (K.D.R.); nahdia.afiifah@ukm.edu.my (N.A.A.J.); tatykamarudin@ukm.edu.my (T.A.K.)

\* Correspondence: fairusahmad@ukm.edu.my; Tel.: +60-39-1458632

**Abstract:** A corneal abrasion results from the disruption or loss of cells in the corneal epithelium. If inadequately treated, it can compromise visual clarity. The wound healing process of a corneal abrasion involves epithelial migration, proliferation and adhesion. *Clitoria ternatea* flower extract (CTE) is rich in flavonoids, anthocyanins and other bioactive compounds. It has antioxidant, anti-inflammatory and wound-healing properties. This study explores the potential of CTE to be used as a natural supplement to improve corneal wound healing. Phytochemical profiling via LC–MS identified a total of 51 distinct bioactive constituents. The anthocyanin content, quantified in terms of cyanidin-3-glucoside equivalent, was quantified at 33.06 mg per gram of extract. The extract exhibited 33.8% DPPH radical scavenging activity and a total polyphenol content equivalent to 24.14 mg/g gallic acid. Human telomerase-immortalized corneal epithelial (hTCEpi) cells maintained in keratinocyte basal medium were utilized to determine cytotoxicity and wound-healing effects. The optimal extract concentration of 0.08 mg/mL, quantified via MTT assay, resulting in enhanced cell viability. Scratch assays demonstrated a higher percentage of wound closure in the CTE-treated group at 6 and 12 h relative to the untreated group, with statistical significance ( $p < 0.05$ ). The gene expressions of CK3 and Cx43, quantified via qRT-PCR, showed no significant differences between groups. However, within the CTE-treated group, CK3 expression increased at 12 h relative to 0 h and 6 h, and Cx43 expression rose significantly at 12 h compared with 0 h ( $p < 0.05$ ). Immunofluorescence confirmed positive protein expression of both markers. These findings suggest that CTE possesses potent antioxidant properties and promotes corneal epithelial wound healing through upregulation of CK3 and Cx43 in vitro.

**Keywords:** *Clitoria ternatea*; corneal epithelial cells; antioxidant activity; phytochemicals; cell migration

## 1. Introduction

The cornea is the eye's primary refractive surface, comprising five layers that ensure optical clarity and serve as a protective barrier [1]. Limbal epithelial stem cells, located at the corneoscleral junction, enable continuous regeneration of the epithelium to maintain corneal transparency [2].

Corneal abrasion is a common ocular injury caused by trauma, foreign bodies, and improper contact lens use [3]. Patients often present with pain, redness, tearing, and photophobia, and central abrasions can impair vision [4]. Untreated abrasions may progress to



ulcers, microbial keratitis, or stromal scarring, leading to permanent visual impairment [5]. In Malaysia and other Southeast Asian countries, occupational and environmental risks contribute to the high burden of corneal abrasions [6].

Conventional management includes lubricants, topical antibiotics, and analgesics; however, current therapies are limited, and adjunctive treatments that accelerate epithelial recovery are still needed [7]. Natural products such as omega-3 fatty acids and Manuka honey have demonstrated potential to improve wound healing outcomes [8]. For instance, 0.025% Acacia honey promotes the proliferation of corneal epithelial cells while maintaining their normal phenotype and enhancing the expression of key wound healing proteins [9]. Similarly, *Centella asiatica* aqueous extract has been found to stimulate cell proliferation and migration of corneal epithelial cells, suggesting its role in accelerating wound healing [10].

At the molecular level, cytokeratin 3 (CK3) and connexin 43 (Cx43) are important markers of corneal epithelial homeostasis and wound healing. CK3 contributes to epithelial integrity [11], while Cx43 regulates intercellular communication during tissue repair [12].

*Clitoria ternatea* (butterfly pea flower) is rich in anthocyanins, flavonoids, and cyclotides that exhibit antioxidant, anti-inflammatory, and antimicrobial activities [13]. Prior studies have shown its wound-healing potential in diabetic animal models [7]. However, little is known about its role in corneal epithelial biology, particularly its effects on corneal epithelial cell proliferation, migration, and wound healing. This study therefore investigates the ability of CTE to enhance corneal epithelial cell proliferation, migration, and wound healing. By linking its phytochemical profile to cellular outcomes, this work addresses a critical gap in the development of natural adjuvant therapies for corneal injury.

## 2. Results

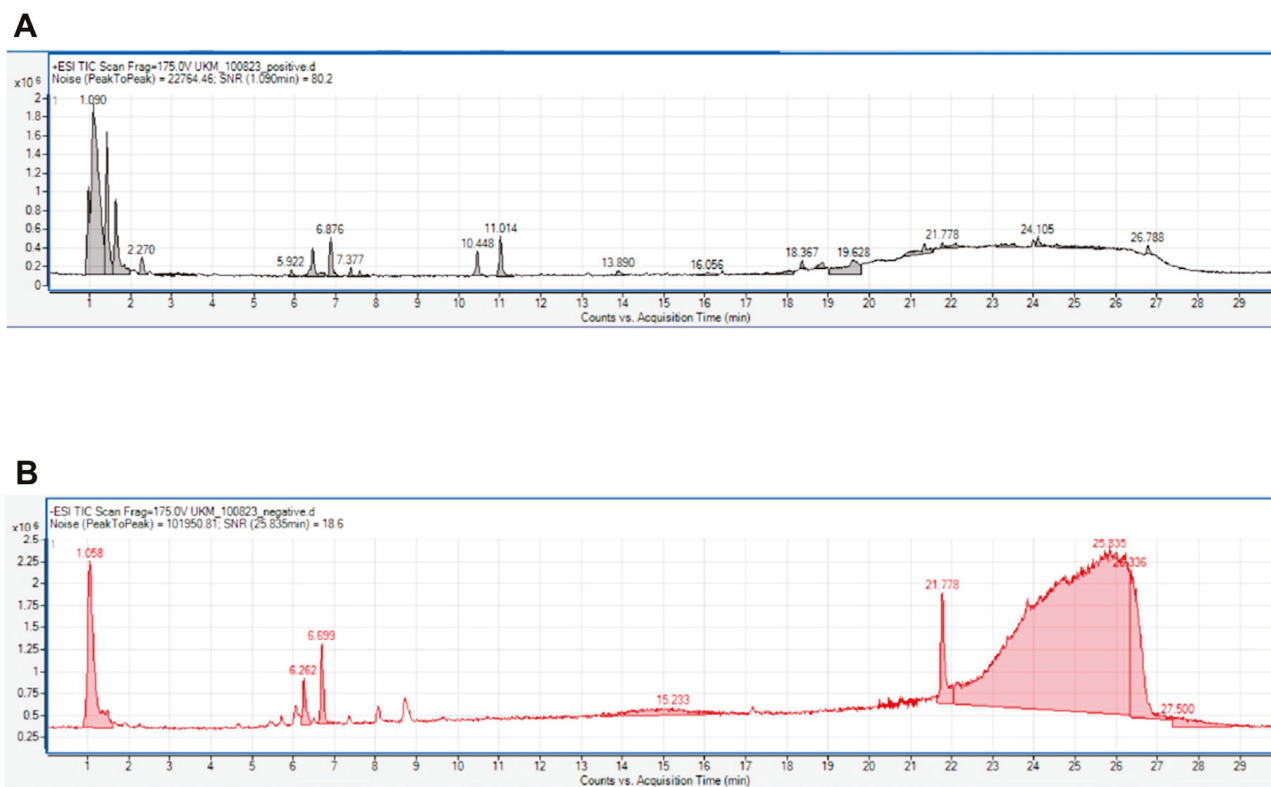
### 2.1. Phytochemical Analysis of CTE

#### 2.1.1. Phytochemical Profiling

LC-MS analysis revealed the presence of various phytochemical constituents in CTE. The total ion chromatogram (TIC) showed distinct peak profiles under both positive and negative ionization modes (Figure 1). The LC-MS analysis revealed 14 peaks in the chromatogram acquired under positive ionization mode and 8 peaks under negative ionization mode, indicating the presence of multiple phytochemical constituents in the CTE. A total of 51 phytochemicals were tentatively identified based on matching retention times and  $m/z$  values with the LC-MS database. The predicted compounds were annotated using the METLIN metabolomics database (Agilent Technologies, Santa Clara, CA, USA) in combination with Agilent Mass Hunter Qualitative Analysis B.05.00 software. Compound identification was based on accurate mass matching and should therefore be considered tentative in the absence of authentic reference standards or fragmentation analysis.

The identified compounds include flavonoids, anthocyanins, fatty acids, alkaloids, terpenoids, steroids, and other antioxidant-related groups. Notable examples include delphinidin, kaempferol derivatives, luteolin glycosides, and auranthidin. Although an aqueous extraction was used, the presence of lipophilic compounds such as fatty acids and terpenoids may be explained by their partial solubility at elevated temperatures (50 °C) or by co-extraction of trace amounts. This possibility has been acknowledged as a limitation. Table 1 summarizes the antioxidant group and its representative compounds. The complete list of 51 phytochemicals is provided in Supplementary Tables S1 and S2.





**Figure 1.** The retention times of the compound peaks produced by chromatography of CTE flower extract. The retention times of compounds formed in positive (A) and negative (B) ionization modes are shown in the figure above.

**Table 1.** Quantification of phenolics, flavonoids, and antioxidant properties in CTE flower extract.

| Group        | Number of Compounds | Representative Examples                              |
|--------------|---------------------|------------------------------------------------------|
| Flavonoids   | 7                   | Kaempferol, Luteolin, Scutellarein                   |
| Anthocyanins | 6                   | Delphinidin, Pelargonidin, Cyanidin                  |
| Fatty acids  | 7                   | 9(S)-HpODE, Hydroxy-octadecanoic acids               |
| Alkaloids    | 3                   | Vernoflexuoside, Isokarabostiril                     |
| Terpenoids   | 2                   | Tiarubrin A, Ectocarpin                              |
| Peptides     | 1                   | Thr-Phe                                              |
| Others       | 10+                 | Includes vitamin analogs, phosphates, prostaglandins |

#### 2.1.2. Anthocyanin Content

The anthocyanin concentration, measured as cyanidin-3-glucoside equivalent, was 33.06 mg/g of extract, equivalent to 3.3% anthocyanin content in CTE.

#### 2.1.3. Antioxidant Activity (DPPH Assay)

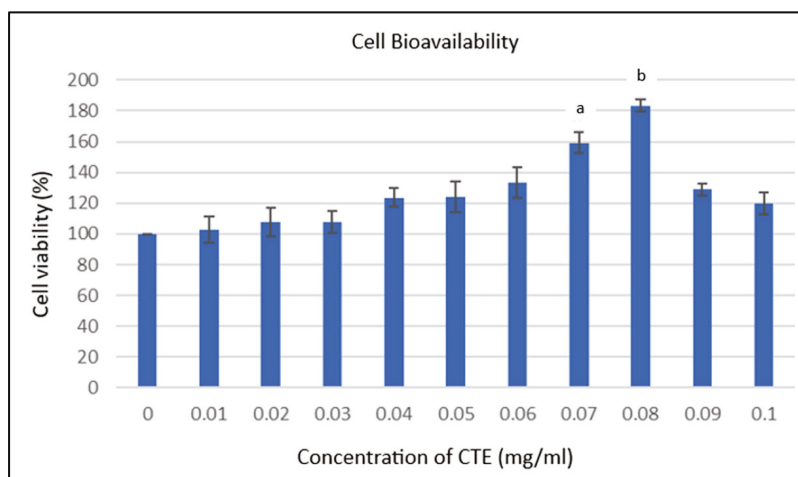
The DPPH radical scavenging activity of CTE was 33.8% at 0.1 g/mL, which is lower than that of the synthetic antioxidant BHA (95%) and the natural standard ascorbic acid (92%) at equivalent concentrations. Based on commonly used classifications, this level of scavenging activity is considered modest.

#### 2.1.4. Total Polyphenol Content

By the Folin–Ciocalteu method, the total phenolic content was quantified at 24.14 mg gallic acid equivalents (GAE) per gram of extract.

## 2.2. Determination of Optimal Dose for Cell Viability

The MTT assay revealed that 0.08 mg/mL showed the highest cell viability ( $183.34 \pm 3.70\%$ ) within the 72 h timeframe tested. Statistically significant increases in viability were observed at 0.07 and 0.08 mg/mL compared with lower and higher concentrations (a and b,  $p < 0.05$ ; Figure 2). This optimal dose was used in all subsequent experiments.



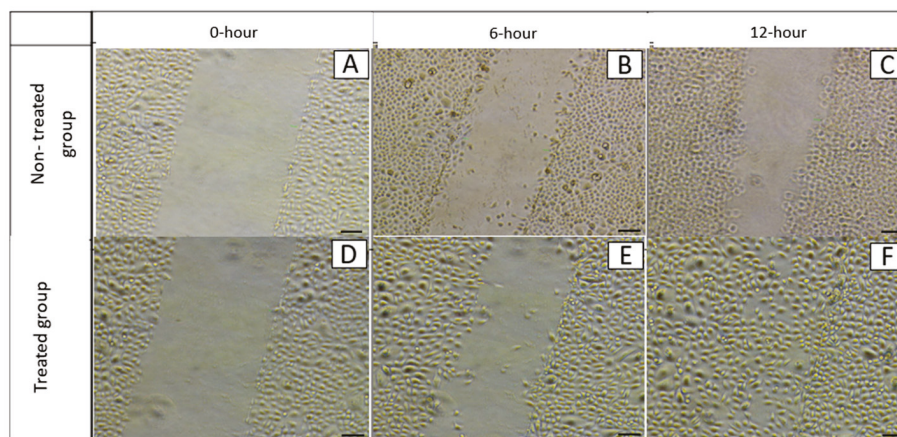
**Figure 2.** Percentage of hTCEpi cell viability on day three of culture. hTCEpi cells were treated with serial dilutions of CTE at graded concentrations (0.01–0.1 mg/mL). Values represent mean  $\pm$  standard error of the mean (S.E.M),  $n = 6$ . Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. a indicates a significant difference ( $p < 0.05$ ) compared with treatment concentrations from 0 to 0.05 mg/mL and 0.1 mg/mL. b Indicates significant compared to treatment doses from 0 to 0.06 mg/mL and 0.09 to 0.1 mg/mL of *Clitoria ternatea* extract ( $p < 0.05$ ).

## 2.3. Effect of CTE on hTCEpi Cell Migration

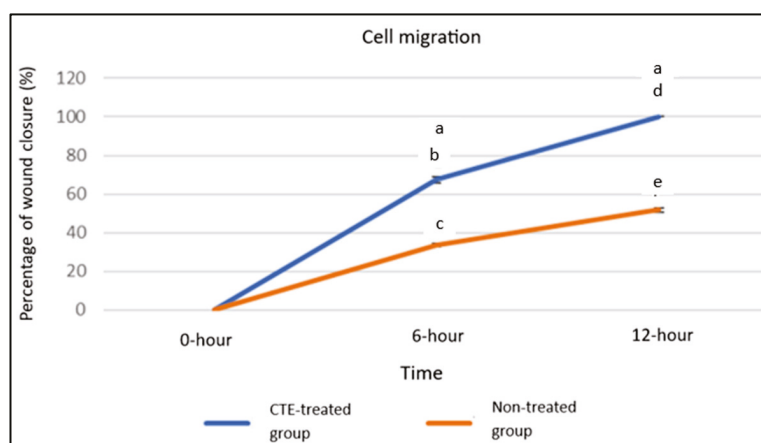
The wound healing model of cell scratch assay demonstrated that treatment with 0.08 mg/mL CTE significantly enhanced wound closure compared to control. Complete wound closure (100%) was observed after 12 h in the treated group (F), whereas the untreated group achieved only  $51.83 \pm 1.11\%$  closure (C) (a,  $p < 0.05$ ) (Figures 3 and 4). At 6 h, the treated group showed  $67.50 \pm 1.58\%$  wound closure, while the untreated group exhibited  $33.67 \pm 0.71\%$  closure (a,  $p < 0.05$ ) (Figure 4). Additionally, wound closure increased significantly over time at 0, 6, and 12 h within both the CTE-treated group (b and d,  $p < 0.05$ ) and the groups that are not treated (c and e,  $p < 0.05$ ) (Figure 4).

## 2.4. Gene Expression Analysis (qRT-PCR)

qRT-PCR analysis indicated no statistically significant differences between treated and untreated groups for CK3 or Cx43 at equivalent time points. However, within the treated group, CK3 expression was markedly higher at 12 h compared with both 0 and 6 h (a,  $p < 0.05$ ). In untreated cultures, CK3 levels also increased significantly at 12 h relative to baseline (b,  $p < 0.05$ ). For Cx43, expression rose significantly in treated cells at 12 h versus 0 h (a,  $p < 0.05$ ) (Figures 5 and 6).



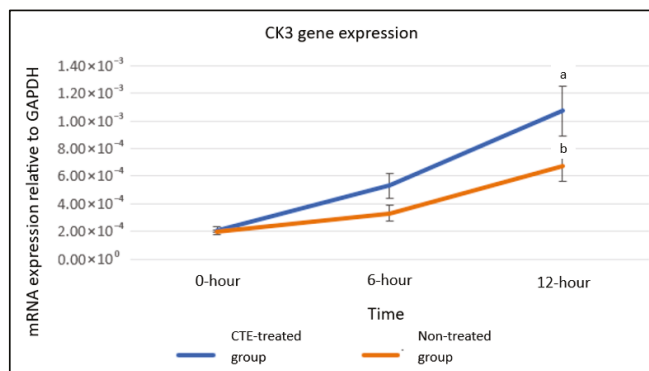
**Figure 3.** Photomicrographs showing the wound area and hTCEpi cell migration captured using time-lapse microscopy at the beginning of the experiment (0-h), after 6 h (6-h), and after 12 h (12-h), with  $n = 6$ . The in vitro hTCEpi wound model without treatment is shown in photomicrographs (A–C), while the model treated with 0.08 mg/mL CTE flower extract is shown in photomicrographs (D–F). Magnification:  $\times 10$ . Scale bar = 100  $\mu\text{m}$ .



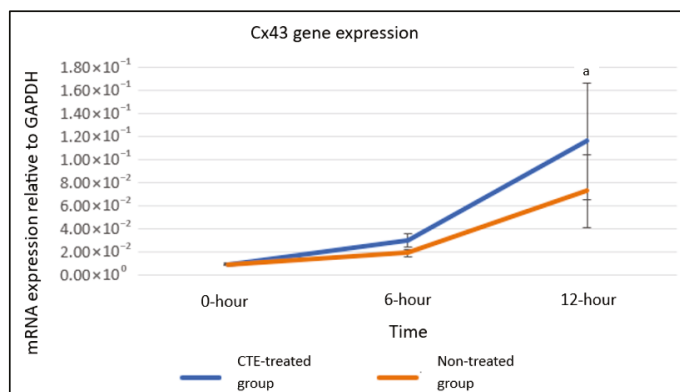
**Figure 4.** Percentage of wound closure in the hTCEpi cell migration assay for cultures without treatment and with 0.08 mg/mL CTE flower extract treatment. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test and are expressed as mean  $\pm$  standard error of the mean (S.E.M), with  $n = 6$ . a: Significant compared to untreated hTCEpi cells at 6 and 12 h ( $p < 0.05$ ). b: Significant compared to treated hTCEpi cells at 0 h. c: Significant compared to untreated hTCEpi cells at 0 h. d: Significant compared to treated hTCEpi cells at 0 and 6 h. e: Significant compared to untreated hTCEpi cells at 0 and 6 h.

## 2.5. Protein Expression via Immunofluorescence

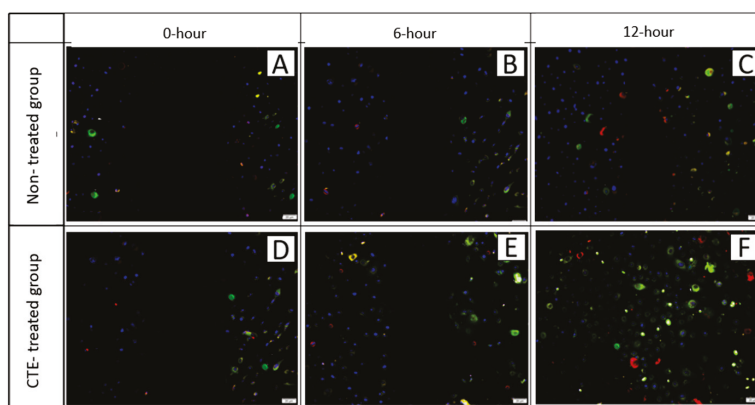
Immunofluorescence staining confirmed expression of CK3 and Cx43 proteins in hTCEpi cells. CK3 was localized in the cytoplasm and membrane (green), while Cx43 appeared as red fluorescence in the cytoplasm. Nuclear staining with DAPI (blue) verified cellular morphology. Cells treated with 0.08 mg/mL CTE showed positive protein expression of CK3 and Cx43 at the wound margins (D and E) and within the wound at 12 h (F) post-treatment (Figure 7).



**Figure 5.** CK3 gene expression in hTCEpi cells cultured without treatment and with 0.08 mg/mL CTE flower extract treatment. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test and are expressed as mean  $\pm$  standard error of the mean (S.E.M), with  $n = 6$ . a: Significant compared to hTCEpi cells treated with 0.08 mg/mL CTE at 0 and 6 h ( $p < 0.05$ ). b: Significant compared to untreated hTCEpi cells at 0 h ( $p < 0.05$ ).



**Figure 6.** Cx43 gene expression in hTCEpi cells cultured without treatment and with 0.08 mg/mL CTE flower extract treatment. Data were analyzed using two-way ANOVA and Tukey's post hoc test, and results are presented as mean  $\pm$  standard error of the mean (S.E.M), where  $n = 6$ . a: significant compared to hTCEpi cells treated with 0.08 mg/mL CTE at 0 h ( $p < 0.05$ ).



**Figure 7.** Photomicrographs showing immunofluorescence staining of CK3 (green) and Cx43 (red) proteins at the wound margins in vitro, representing one-sixth of the full hTCEpi cell monolayer at 0 h, 6 h, and 12 h. CK3 protein (green) is indicated by green-stained cytoplasm, while Cx43 protein (red) is indicated by red-stained membranes and cytoplasm. Cell nuclei (blue) are stained in blue. Cells were non-treated (A–C) and treated with 0.08 mg/mL CTE (D–F). Magnification:  $\times 100$ . Scale bar = 100  $\mu\text{m}$ .

### 3. Discussion

The LC–MS analysis confirmed that CTE contains diverse bioactive compounds, including flavonoids, anthocyanins, alkaloids, fatty acids, terpenoids, phospholipids, and vitamin D analogs [14,15]. Flavonoids such as kaempferol and luteolin are known to reduce oxidative stress and modulate inflammatory pathways, both of which are implicated in epithelial proliferation and repair [16]. Anthocyanins, including cyanidin and delphinidin, can stimulate collagen synthesis and cell migration, processes directly relevant to wound closure in corneal epithelium [17]. These bioactive compounds may exert their effects through a combination of antioxidant, anti-inflammatory, and cytoprotective mechanisms, all of which are critical for promoting tissue regeneration in the cornea. Fatty acids and alkaloids may provide additional antimicrobial and anti-inflammatory support that could contribute to corneal defense and regeneration [18]. It should be noted that the detection of lipophilic compounds from an aqueous extract may reflect partial solubility at higher extraction temperatures or minor co-extraction. This highlights a methodological limitation and should be explored in future extraction optimization studies.

Interestingly, the anthocyanin content obtained in this study (33.06 mg/g) was higher than previously reported [19]. This difference may be due to the use of aqueous extraction, which enhances compound yield compared to solvent-based methods [20]. Given the role of anthocyanins in antioxidant defense and tissue remodeling, higher yields may increase the biological potency of CTE for ocular applications [21]. The presence of these compounds suggests that CTE may help mitigate oxidative damage, modulate inflammation, and stimulate wound healing in the corneal epithelium, making it a promising candidate for ocular injury treatment [22].

The DPPH assay demonstrated that CTE possessed a scavenging capacity of 33.8%, which is modest compared with synthetic antioxidants such as BHA and natural standards such as ascorbic acid [23]. Nevertheless, even moderate antioxidant activity may be physiologically meaningful, as oxidative stress is known to impair corneal epithelial wound healing [24]. In the context of corneal injury, reducing oxidative damage, even modestly, could contribute to a more favorable healing environment. Moreover, antioxidant assays alone do not capture the synergistic activity of phytochemicals; thus, the observed enhancement of proliferation and migration *in vitro* may result from combined effects of multiple compounds present in CTE, such as flavonoids, anthocyanins, and fatty acids, which collectively enhance cellular responses to injury through antioxidative, anti-inflammatory, and proliferative pathways [21].

The MTT assay showed that 0.08 mg/mL produced the highest cell viability within the 72 h timeframe tested. However, this concentration should not be considered “optimal” without further validation, as the assay used a narrow concentration range and a single readout. Such biphasic effects are common with polyphenols, where lower concentrations stimulate proliferation, but higher concentrations may induce cytotoxicity [20]. Future studies should extend the dose–response range and include positive controls such as epidermal growth factor (EGF) for proliferation or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for cytotoxicity [25].

The wound-healing assay confirmed faster closure in CTE-treated cells, consistent with enhanced proliferation and migration. CK3 expression increased at 12 h, reflecting its role in epithelial differentiation during healing [22], while Cx43 expression was also upregulated, indicating active intercellular signaling at the wound margin [23]. Similar effects have been described with other natural agents, including edible bird’s nest and bee venom [26,27]. The observed increase in Cx43 expression may be consistent with facilitation of tissue repair [28]. The upregulation of Cx43 in corneal fibroblasts via insulin-like growth factor-1 (IGF-1) facilitates cell proliferation, migration, and differentiation



during wound healing by enabling intercellular signaling such as MAPK/ERK pathway. This communication helps coordinate the healing process, ensuring proper regeneration of the epithelium [29]. Elevated Cx43 in later stages supports extracellular matrix remodeling, fibroblast activation, and angiogenesis, all critical for reinforcing and stabilizing healed corneal tissue [30].

Despite the absence of a positive control such as growth factor, the results from the scratch assay still provide meaningful insights as the treated group demonstrated significant cell proliferation and migration, indicating a clear improvement in wound healing. Although CK3 and Cx43 expression levels appeared elevated at 12 h in the CTE-treated group, these differences were not statistically significant compared with the untreated controls, which also showed increased expression over time. Therefore, the present findings cannot confirm a direct effect of CTE on these molecular markers. While the trends observed may be consistent with known roles of CK3 in epithelial differentiation and Cx43 in intercellular communication, further studies incorporating positive controls, additional replicates, and *in vivo* validation are required to determine whether CTE exerts a true modulatory effect on these proteins. In addition, between 0 h and 6 h, the treatment group showed a significantly higher rate of CK3 and Cx43 expression compared to the control group, suggesting that the treatment stimulates a stronger or more rapid differentiation response during the early stages of corneal wound healing.

This study suggests that CTE holds potential as a therapeutic agent for corneal epithelial injuries. Its bioactive compounds such as flavonoids, anthocyanins, and alkaloids are known to regulate oxidative stress, inflammation, and cellular proliferation, which are critical for wound healing [31]. The observed improvement in cell proliferation, migration, and wound closure indicates that CTE may promote corneal regeneration, making it a potential adjunct in treating corneal injuries or disorders.

To translate these findings into clinical practice, further studies are necessary to optimize the formulation and delivery of CTE, potentially through eye drops or gels, to ensure effective application to the ocular surface. Additionally, *in vivo* studies using animal models are needed to evaluate the safety and efficacy of CTE in a more complex biological context. Incorporating positive controls such as EGF and investigating its effects on molecular markers like CK3 and Cx43 will be essential for confirming its therapeutic potential. These studies, combined with further exploration of its molecular mechanisms, will facilitate the development of CTE as a viable natural therapy for corneal epithelial injury and other ocular conditions.

#### 4. Limitations

This study has several limitations that should be acknowledged. First is the absence of detailed cultivation site information for the plant material. Although authentication was performed and a voucher specimen deposited, future studies should ensure full traceability of plant sources, including cultivation site and geographic coordinates. The phytochemical profile through METLIN database was performed without authentic standards or MS/MS fragmentation should therefore be regarded as preliminary, and future studies should include reference standards and quantitative analyses to confirm compound identities and relative abundance.

The MTT assay was conducted within a relatively narrow concentration range (0.01–0.1 mg/mL). Although this range was selected based on preliminary pilot studies and solubility constraints, a broader dose–response analysis and additional time points are needed to confirm whether 0.08 mg/mL represents an optimal concentration or simply the most effective under the conditions tested.



There are no positive controls included in the assays. In particular, epidermal growth factor (EGF) could serve as a proliferation and wound-healing benchmark, while hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) could act as a cytotoxic comparator.

Similarly, the scratch assay compared only untreated and CTE-treated cells, without a positive healing control. The absence of these controls limits direct comparison with established modulators of corneal wound healing.

Finally, the findings are restricted to an immortalized corneal epithelial cell line in an in vitro setting. Future studies should therefore incorporate positive controls, primary human corneal epithelial cells, in vivo validation, and comparison with standard therapeutic agents to strengthen the translational relevance of CTE.

## 5. Materials and Methods

Ethical approval for this research was obtained from the Universiti Kebangsaan Malaysia Research Ethics Committee (Reference: JEP-2023-563) on 24 August 2023. All experimental work complied with the institutional guidelines for ethical conduct in research.

### 5.1. Plant Material and Extract Preparation

Fresh flowers of *Clitoria ternatea* were purchased from a supplier in Petaling Jaya, Malaysia. Precise cultivation site details and geographic coordinates were not available from the supplier. To ensure accurate identification, the plant material was authenticated by the Herbarium, Universiti Kebangsaan Malaysia (UKMB), and a voucher specimen was deposited under the accession number ID081/2023. The flowers were processed using an aqueous extraction method, where they were blended with DNA/RNA-free distilled water (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a 1:15 (*w/v*) proportion. The mixture was incubated at 50 °C for 30 min, filtered, freeze-dried, and stored at 8 °C. A stock solution was prepared at a concentration of 10 mg/mL.

### 5.2. Phytochemical Analysis of CTE

#### 5.2.1. Phytochemical Profiling

LC–MS analysis was carried out using an ACQUITY I-Class Ultra-High-Performance Liquid Chromatography (UHPLC) system (Waters Corporation, Milford, Massachusetts, USA) equipped with a binary pump, autosampler, vacuum degasser, and column oven. Separation of phenolic compounds was achieved on an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm × 1.8 µm) maintained at 40 °C. The mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B), with a binary linear gradient at a flow rate of 0.6 mL/min. The injection volume was set to 1 µL. The UHPLC system was connected to a Vion IMS QTOF hybrid mass spectrometer (Waters Corporation, Milford, Massachusetts, USA) using a LockSpray ion source in positive and negative electrospray ionization modes. LC–MS analysis was performed using both positive (ESI+) and negative (ESI−) electrospray ionization modes to ensure broader metabolite coverage. Parameters included a capillary voltage of 1.50 kV, reference capillary voltage of 3.00 kV, source temperature of 120 °C, desolvation temperature of 550 °C, desolvation gas flow at 800 L/h, and cone gas flow at 50 L/h. Nitrogen (>99.5%) was used for both desolvation and cone gases. Data acquisition was performed in high-definition MSE (HDMSE) mode over an *m/z* range of 50–1500 with a scan interval of 0.1 s.

#### 5.2.2. Anthocyanin Content

Anthocyanin levels were quantified at the UKM-MTDC Technology Centre, Universiti Kebangsaan Malaysia, using an HPLC system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following a modified method [14]. The chromatographic column was identical to that

used for LC–MS. The content was expressed as cyanidin-3-glucoside equivalents (mg/g) by comparison with a standard. Two mobile phases were prepared: Solvent A—trifluoroacetic acid in deionized water (pH 2.5) and Solvent B—100% methanol. A gradient program was applied as follows: 0 min, 100% A; 20 min, 50% A/50% B; 30 min, 100% B; and 35–40 min, 100% A. Detection was set at 280 nm with a 20 µL injection volume. Extraction involved hydrolyzing 1 g of CTE with a mixture of 12 mL methanol, 8 mL deionized water, and 5 mL 6 M HCl at 95 °C for 2 h, followed by cooling, filtration through a 0.45 µm Whatman nylon membrane (Waters GmbH, Eschborn, Hessen, Germany), and analysis.

### 5.2.3. Antioxidant Activity (DPPH Assay)

The antioxidant potential of CTE was measured using the DPPH radical scavenging method at the UKM-MTDC Technology Centre [32]. A 100 µL aliquot of extract at concentrations between 200 and 1000 µg/mL was mixed with 3.9 mL DPPH solution (25 mg/L in aqueous medium) and kept in the dark for 30 min. Absorbance was recorded at 515 nm. Methanol served as a blank, the positive control consisted of methanol with DPPH, and butylated hydroxyanisole (BHA) was used as the reference antioxidant. Results were expressed as the percentage of DPPH radical inhibition using:

$$\% \text{ Inhibition} = [( \text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}} ) \div \text{Absorbance}_{\text{Control}}] \times 100$$

### 5.2.4. Total Polyphenol Content

Polyphenol content was determined via the Folin–Ciocalteu assay [33]. In brief, 100 µL ethanolic extract (1 mg/mL) was combined with 7.9 mL distilled water and 0.5 mL Folin–Ciocalteu reagent. After 2 min, 1.5 mL 7.5% sodium carbonate was added, and the mixture was incubated for 2 h at room temperature. Absorbance was measured at 765 nm (Nakagyo-ku, Kyoto, Japan). Values were expressed as gallic acid equivalents (mg GAE/g dry extract).

### 5.3. Cell Culture

The hTCEpi cell line was expanded, which we obtained from Evercyte. Human telomerase-immortalized corneal epithelial (hTCEpi) cells were maintained under sterile culture conditions. Cells stored in liquid nitrogen (−196 °C) were thawed rapidly in a 37 °C water bath and transferred to a 15 mL centrifuge tube containing 9 mL of Keratinocyte Basal Medium (KBM; Lonza, Morristown, New Jersey, USA). Following centrifugation at 170× g for 5 min at room temperature, the resulting pellet was gently resuspended in 1 mL of fresh KBM. The cell suspension was seeded into T25 flasks pre-equilibrated with 3 mL KBM. Cultures were incubated at 37 °C in 5% CO<sub>2</sub>, with the first medium change after 24 h and subsequently every 48 h. When cells reached 70–80% confluence, subculturing was performed using 0.05% EDTA. Flasks were rinsed with phosphate-buffered saline (PBS) and treated with 2 mL EDTA for 2.5 min at 37 °C to detach cells, followed by gentle tapping. A second detachment step with another 2 mL EDTA for 1.5 min was performed to ensure complete release. Cells were pooled, centrifuged at 170× g for 5 min, the supernatant discarded, and the pellet resuspended in 4 mL KBM. Viability was assessed via trypan blue exclusion (90 µL cell suspension mixed with 10 µL trypan blue), and viable cells were counted using a hemocytometer under an inverted microscope. Passages up to 11 were used for downstream experiments, including migration assays, gene expression analysis, and immunofluorescence.

### 5.4. MTT Assay for Cytotoxicity and Optimal Dose

For cytotoxicity evaluation and dose optimization, hTCEpi cells ( $5 \times 10^4$  cells/well) were seeded into 96-well plates and treated with CTE at concentrations ranging from 0.01 to 0.1

mg/mL. After 72 h, 10 µL of MTT reagent (5 mg/mL) was added to each well and incubated for 4 h. Formazan crystals were dissolved using DMSO, and absorbance was measured at 570 nm. The concentration showing the highest cell viability was selected for subsequent experiments. This assay was limited by the use of a relatively narrow concentration range (0.01–0.1 mg/mL). Although this range was selected based on preliminary pilot studies and solubility constraints, a broader dose–response analysis would provide a more comprehensive understanding of the cytotoxic and proliferative effects of CTE. In addition, positive controls such as epidermal growth factor (EGF) for proliferation or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for cytotoxicity were not included. The absence of these controls represents a limitation of the experimental design and should be addressed in future studies.

### 5.5. Cell Scratch Assay

Cell scratch assays were performed on confluent hTCEpi monolayers. A vertical scratch was made using a 200 µL pipette tip. Cells were divided into control and CTE treatment groups. Wound healing was imaged at 0, 6, and 12 h. Wound closure (%) was calculated using the formula:

$$[G(0) - G(t)]/G(0) \times 100\%.$$

### 5.6. RNA Extraction and qRT-PCR

Total RNA was isolated from cells using TRI reagent, and concentration and purity were assessed with a NanoDrop spectrophotometer. Complementary DNA (cDNA) synthesis was performed with LunaScript RT SuperMix (New England Biolabs, Ipswich, Massachusetts, USA). Quantitative real-time PCR (qRT-PCR) was carried out using Luna Universal qPCR Master Mix with primers specific for CK3, Cx43, and GAPDH. Gene expression was calculated using the 2<sup>ΔCt</sup> method.

### 5.7. Immunofluorescence Staining

Fixation of cells carried out with 4% paraformaldehyde, permeabilized and blocked. Primary antibodies against CK3 and Cx43 were applied, followed by fluorescent secondary antibodies. Nuclei were counterstained with DAPI. Fluorescent images were obtained using a fluorescence microscope.

### 5.8. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 9 software. Parametric datasets were analyzed using two-way ANOVA followed by Tukey's post hoc test, while non-parametric data were evaluated using the Kruskal–Wallis test and Mann–Whitney U test. Results are presented as mean ± standard error of the mean (SEM), with *p*-values < 0.05 considered statistically significant.

## 6. Conclusions

This study demonstrates that *Clitoria ternatea* flower extract (CTE) enhances corneal epithelial cell proliferation and migration in vitro and modulates CK3 and Cx43 expression, suggesting a potential role in supporting epithelial repair. The phytochemical analysis confirmed the presence of bioactive compounds, including flavonoids and anthocyanins, which may contribute to these observed effects through antioxidant and wound-healing mechanisms.

However, these findings remain preliminary and are limited to an immortalized corneal epithelial cell line without the inclusion of positive controls or in vivo validation. Future studies should incorporate primary human corneal cells, appropriate proliferation and wound-healing controls, and animal models to confirm the therapeutic relevance of

CTE. Comparative studies with standard therapeutic agents will also be necessary before considering its clinical translation as an adjunctive treatment for corneal injury.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants14203216/s1>, Table S1: List of identified phytochemicals and their molecular weights (positive ionization mode); Table S2: List of identified phytochemicals and their molecular weights (negative ionization mode).

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## Abbreviations

The following abbreviations are used in this manuscript:

|         |                                                                             |
|---------|-----------------------------------------------------------------------------|
| CTE     | <i>Clitoria ternatea</i> flower extract                                     |
| LC-MS   | Liquid Chromatography–Mass Spectrometry                                     |
| DPPH    | 2,2-Diphenyl-1-picrylhydrazyl                                               |
| GAE     | Gallic Acid Equivalent                                                      |
| hTCEpi  | Human Telomerase-Immortalized Corneal Epithelial Cells                      |
| KBM     | Keratinocyte Basal Medium                                                   |
| MTT     | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide                |
| CK3     | Cytokeratin 3                                                               |
| Cx43    | Connexin 43                                                                 |
| qRT-PCR | Quantitative Reverse Transcription Polymerase Chain Reaction                |
| DAPI    | 4',6-Diamidino-2-Phenylindole                                               |
| GFFP    | Geran Fundamental Fakulti Perubatan (Fundamental Faculty of Medicine Grant) |
| PBS     | Phosphate-Buffered Saline                                                   |
| DMSO    | Dimethyl Sulfoxide                                                          |
| UHPLC   | Ultra-High Performance Liquid Chromatography                                |
| TIC     | Total Ion Chromatogram                                                      |
| ESI     | Electrospray Ionization                                                     |
| HPLC    | High-Performance Liquid Chromatography                                      |
| HDMSE   | High-Definition Mass Spectrometry Elevated Energy                           |
| IGF-1   | Insulin-like Growth Factor-1                                                |
| Anova   | Analysis of variance test                                                   |

|       |                                            |
|-------|--------------------------------------------|
| DMEK  | Descemet membrane endothelial keratoplasty |
| EDTA  | Ethylenediaminetetraacetic acid            |
| mg    | milligram                                  |
| ml    | milliliter                                 |
| mm    | millimeter                                 |
| MMPs  | Matrix metalloproteinases                  |
| nm    | nanometer                                  |
| PFA   | Paraformaldehyde                           |
| S.E.M | Standard error of the mean                 |
| µm    | Micrometer                                 |

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## Article

# The Impact of Temperature on the Leaves of *Ceratonia siliqua* L.: Anatomical Aspect, Secondary Metabolite Analysis, and Antimicrobial Activity of the Extracts

Aikaterina L. Stefi <sup>1,\*</sup>, Evangelos Kalampokis <sup>2</sup>, Georgia C. Ntroumpogianni <sup>1</sup>, Iliana Katsiadrami <sup>1</sup>, Theodora Nikou <sup>2</sup>, Efsthios A. Katsifas <sup>1</sup>, Dimitrios Gkikas <sup>1</sup>, Nikolaos S. Christodoulakis <sup>1</sup> and Maria Halabalaki <sup>2</sup>

<sup>1</sup> Section of Botany, Department of Biology, Faculty of Sciences, National and Kapodistrian University of Athens, 15784 Athens, Greece; georgiantr@biol.uoa.gr (G.C.N.); ili\_ka@hotmail.com (I.K.); skatsi@biol.uoa.gr (E.A.K.); dimgkikas@biol.uoa.gr (D.G.); nchristo@biol.uoa.gr (N.S.C.)

<sup>2</sup> Division of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, 15784 Athens, Greece; kalampokis-v@pharm.uoa.gr (E.K.); th-nikou@pharm.uoa.gr (T.N.); mariahal@pharm.uoa.gr (M.H.)

\* Correspondence: kstefi@biol.uoa.gr

**Abstract:** *Ceratonia siliqua* L. (Fabaceae) is an evergreen sclerophyllous species that successfully overcomes the challenges of the Mediterranean climate. Commonly, biosynthesis of secondary metabolites is a major reaction of the plants thriving in the Mediterranean formations against temperature stress. Due to concerns about the climate crisis, we studied the impact of 6-day low (5 °C) and high (40 °C) temperature stress on young carob seedlings. In stressed plants, mainly the heat-treated, the leaves appear xeromorphic. Parameters of the physiology of the plants such as chlorophyll-a and -b, total phenolic content, and oxidative stress were measured and presented via Principal Component Analysis. Chlorophyll-a and -b contents are inferior in cold-stressed leaves while heat-stressed leaves accumulate more phenolics and experience higher oxidative stress as compared to their cold-stressed counterparts. The phytochemical profile of different extracts obtained from stressed carob leaves was identified so as to gain insight into metabolites produced under stress. Moreover, LC-HRMS/MS metabolomic workflow was utilized for the discovery of biomarkers, over- or under-regulated in stressed conditions. The antimicrobial activity of carob leaf extract fractions was assessed against six human pathogen strains and three phytopathogen bacterial strains. MeOH-H<sub>2</sub>O and dichloromethane (DCM) extracts presented notable activity against *Candida albicans* and *Saccharomyces cerevisiae*, while DCM extracts inhibited the growth of *Erwinia amylovora*. We may conclude that carob tree exposure to temperature stress does not have a significant influence on secondary metabolic pathways.

**Keywords:** carob tree; leaf extracts; environmental stress; ecophysiology; LC-HRMS/MS analyses; bioassays

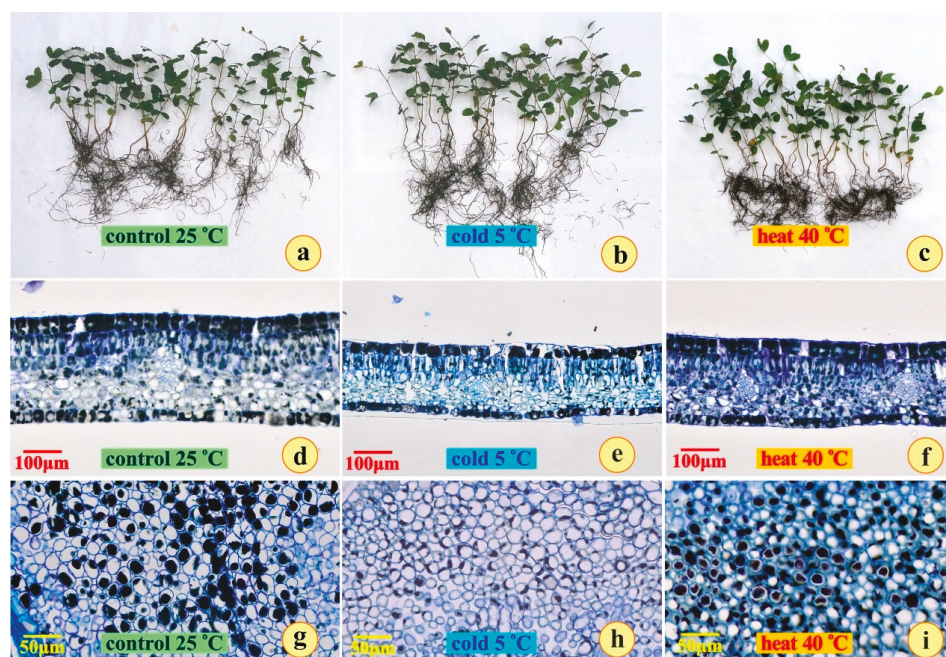
## 1. Introduction

Volumes have been written on the peculiarities of the Mediterranean climate, the evolution, and the adaptation of Mediterranean plants, both the evergreen sclerophylls and the seasonally dimorphic plants. The Mediterranean environment imposes on both plant communities two severe, qualitatively different, and timely separated stresses: the combined stress of drought, high temperature, and high light intensity during the summer

months and freezing temperatures during the winter [1]. Concerning low temperatures, the Mediterranean climate does have a winter, which can readily be defined as any month with an average temperature below 15 °C. However, during the winter, although mild in general, the hours per year that the temperature falls below freezing (0 °C) should not exceed 3% of the total. This phenomenon, being a good index of the severity of winters, directly affects plant life [2].

It has been demonstrated that the distribution of the Mediterranean evergreen sclerophyllous formations is controlled by the winter cold in northern latitudes and the summer drought in southern habitats [3]. Therefore, plants thriving either in the chaparral (evergreen sclerophyllous) or in the phryganic (seasonally dimorphics) formations, have developed interesting adaptive strategies [4] to either evade or escape the above-mentioned environmental stresses.

The carob or locust bean tree (*Ceratonia siliqua* L., Family: Fabaceae) (Figure 1a) is an emblematic species thriving among the evergreen sclerophyll formations [5]. It has developed remarkable adaptations to successfully overcome the overwhelmingly high temperatures and drought occurring during the summer months [4,6]. However, the natural distribution of *C. siliqua* in Greece, compared to other evergreen sclerophyllous species, hardly reaches the latitude of 38°, which is similar to the latitude of the city of Athens, while *Quercus coccifera* is distributed as far as the northern territories of Greece (latitude 39°76') reaching up to 900 m [7]. The same is true for the “flagship” of the Mediterranean formations, the olive tree, which reaches the latitude of 39°64' and an altitude of 500 m [7–9]. These evergreen sclerophylls are present in areas with higher altitudes while they are absent in areas with lower summer drought. This phenomenon is called the “Mediterranean shrub paradox”. This paradox was explained when the distribution data were compared to the cold gradient [3,7] while it was suggested that the limitations in the distribution of *C. siliqua* are mainly due to the susceptibility of this species to low temperatures [7].



**Figure 1.** The plants of *Ceratonia siliqua* after the experiment; (a–c) Photographs of the whole plant for the control (a), cold-stressed (b), and heat-stressed (c) treatments after drying. Cross (d–f) and paradermal (g–i) sections of plastic-embedded leaf, stained with toluidine blue “O” for the control (d,g) cold-stressed (e,h) and heat-stressed (f,i) plants.

In the hostile Mediterranean climate, the periods of favorable conditions, when plant growth can be promoted, are unpredictable and limited [4]. Furthermore, climatic change, one of the main challenges of this era, is accompanied by extreme weather conditions such as high temperatures, prolonged drought periods, severe storms and rainfalls, floods, snowless winters, and cold snap periods that pose immediate pressures on plants [10]. This has an impact not only on plants; the successive impact on crops creates an added new socioeconomic threat. Eventually, Mediterranean plants, although considered to be potentially very productive [11–13], fall short in yield [1,5]. This is due to the stress conditions of not only the long, arid Mediterranean summer but also the rapid “sweep” of the severe chilling conditions during winter. *C. siliqua*, as the distribution data along the Mediterranean basin indicate, seems to be well adapted to the summer stress conditions yet proves rather vulnerable to the winter cold stress (Table 1). There is a strong indication that the young tissues, leaves, and leaf buds exhibit reduced frost resistance [8,9] while the leaf primordia lack any protection scales [14].

**Table 1.** Frost resistance of vegetative organs of adult specimens of Mediterranean sclerophylls and conifers in December and January. Data indicate temperatures (°C) that cause 50% injury. Adapted from [8,9].

| Species                       | Leaves | Leaf Buds | Stem Cambium | Stem Xylem |
|-------------------------------|--------|-----------|--------------|------------|
| <i>Ceratonia siliqua</i>      | −6     | −8        | −9           | −11        |
| <i>Nerium oleander</i>        | −8     | −12       | −14          | −15        |
| <i>Myrtus communis</i>        | −12    | −11       | −17          | −15        |
| <i>Laurus nobilis</i>         | −12    | −10       | −14          | −16        |
| <i>Olea europaea</i>          | −12    | −13       | −20          | −18        |
| <i>Quercus coccifera</i>      | −12    | −13       | −21          | −22        |
| <i>Quercus suber</i>          | −12    | −16       | −26          | −22        |
| <i>Arbutus unedo</i>          | −12    | −17       | −18          | −16        |
| <i>Rhamnus alaternus</i>      | −12    | −18       | −17          | −16        |
| <i>Viburnum tinus</i>         | −13    | −15       | −20          | −17        |
| <i>Pistacia lentiscus</i>     | −14    | −16       | −20          | −17        |
| <i>Quercus ilex</i>           | −15    | −17       | −28          | −26        |
| <i>Phillyrea latifolia</i>    | −16    | −20       | −23          | −22        |
| <i>Pinus pinea</i>            | −13    | −16       | −19          | −17        |
| <i>Pinus halepensis</i>       | −13    | -         | −22          | −18        |
| <i>Cupressus sempervirens</i> | −16    | -         | −29          | −22        |

Plants, in general, respond to a certain environmental stress in various ways. A prominent reaction among Mediterranean plants is the biosynthesis of secondary metabolites. Among them, a major group is that of the phenolic compounds [15–17] produced through the activation of the shikimic acid pathway [18]. Phenylalanine is one of the products of this pathway that, with the addition of malonyl-CoA, results in flavonoid production [19]. The accumulation of phenolics, even as metabolically terminal products, has been considered a very common feature of Mediterranean plants sustaining environmental stress [14,15,20–26]. Moreover, the effect of the stress conditions is also expressed by the decrease of photosynthetic pigment content because of the chloroplast breakdown

caused by active oxygen species [27]. A balance can be reached by numerous secondary metabolites produced through the shikimic acid pathway, which have an antioxidant effect and increase the plant's tolerance to the stress conditions [28,29].

Therefore, (a) willing to exploit the numerous pieces of data on the stress response of a Mediterranean evergreen sclerophyllous shrub; (b) considering that excessively low or high temperatures, which are new conditions in the Mediterranean regions provoked by climatic change, actually impose a severe stress to the plantlets of *C. siliqua*; (c) wondering if the winter cold stress has a similar effect as the summer stress; (d) regarding the shikimic acid pathway as the crucial mechanism for the relief of the oxidative potential of the cells and the origin of some interesting secondary metabolites; and (e) considering the carob tree as a plant of wide distribution and major commercial importance, we undertook this investigation in order to reveal the response of *C. siliqua* young plants after a quick exposure to considerably low and high temperatures and compare their reaction to that of plants used as “controls”. Thus, our primary aim is to detect any reactions concerning the development, morphology, anatomy, and biochemistry of the plant after a short period of stress (heat or cold). Additionally, to delve deeper into the molecular level, an extraction and analysis of the different specimens were performed. The antimicrobial activity against selected plant and human pathogens of the derived extracts was investigated and the most potent were characterized using different analytical platforms.

## 2. Results

The plants of the three groups (control, cold, and heat) were placed on filter paper and left to dry in an oven at 60 °C for three days. Some samples were randomly selected from the three treatments, and the total dry mass (shoot and root) was measured. The three groups appear to have developed similarly during the 6-day temperature stress period (Figure 1a–c). Concerning the anatomy of the leaves, after the observation of numerous sections of the three leaf types, it seems that the leaves of the plants grown as “controls” demonstrate the structure of the tissues common to most Mediterranean evergreen sclerophylls (Figure 1d). The epidermal cells are rich in phenolics. The palisade tissue appears compact both in the cross (Figure 1d) and paradermal (Figure 1g) sections. These leaves are thicker than the leaves of the other two groups. The leaves from the plants grown in the 5 °C chamber appear less xeromorphic (Figure 1e). Their thin epidermal cells, both adaxial and abaxial, and the moderately developed palisade tissue resulted in a thinner leaf (Figure 1e) accumulating less phenolics as well (Figure 1h). The leaves from the plants grown in the 40 °C chamber (Figure 1f) appear more xeromorphic. The epidermal cells are impregnated with densely stained phenolics while the palisade parenchyma, in longitudinal (Figure 1f) and cross sections (Figure 1i) appear very compact. The palisade cells accumulate phenolics (Figure 1h). The cross-sectioned leaves appear thinner than the “control” ones (compare Figure 1d to Figure 1f). Data on the thickness of the leaf tissues are given in Table 2. In particular, the thickness of the adaxial epidermis was statistically different between the cold- and heat-treated samples and the same is true for the total leaf thickness. The adaxial epidermis of the control plants is thicker than the cold-treated ones, while there is no statistically significant difference between the control and heat-treated plants. The total leaf thickness is greater in the control group than in the cold- and heat-treated groups. No statistically significant differences are noticed in the thickness of the abaxial epidermis and the thickness of the palisade parenchyma between the three groups.

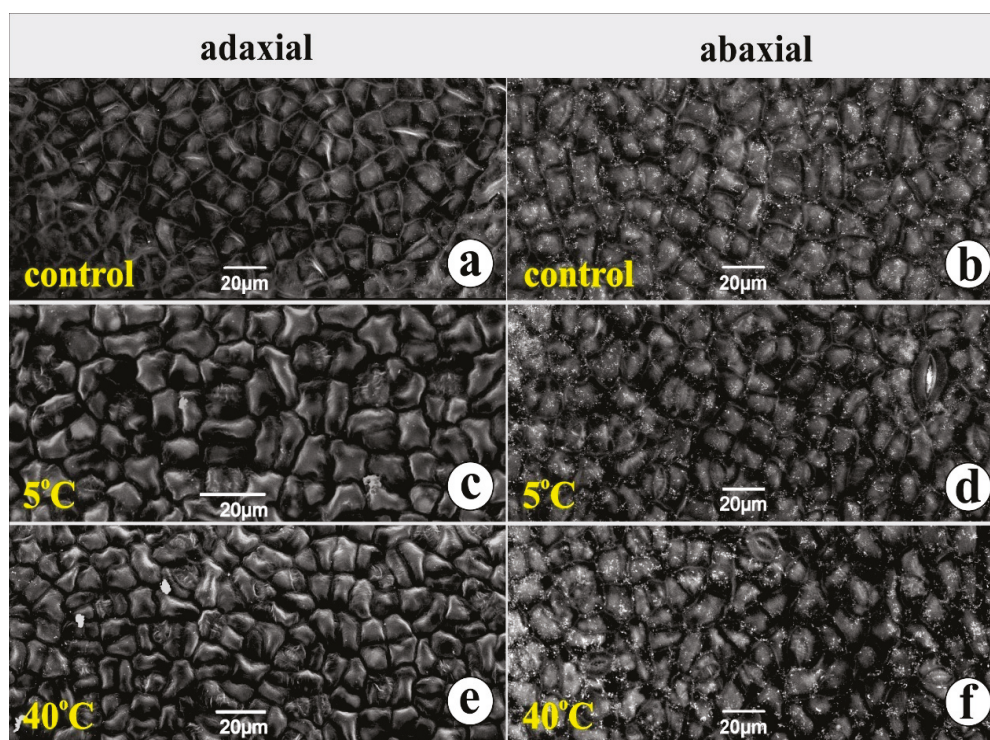
The upper (adaxial) and the lower (abaxial) epidermis of the leaf were thoroughly observed using scanning electron microscopy. Stomata do not exist on the upper epidermis (Figure 2a–c). The lower epidermis of all three leaf types exhibits many small anomocytic



stomata (Figure 2b,d,f). Stomatal frequency was  $234 \pm 22$  for the control plants,  $242 \pm 18$  for the heat-treated plants, and  $225 \pm 12$  for the cold-treated plants, with no statistical difference ( $p > 0.05$ ).

**Table 2.** Data on the thickness of the leaf tissues. (The results with a statistically significant difference are grouped using the compact letter display (CLD) methodology;  $p \leq 0.05$ , according to Tukey test comparisons).

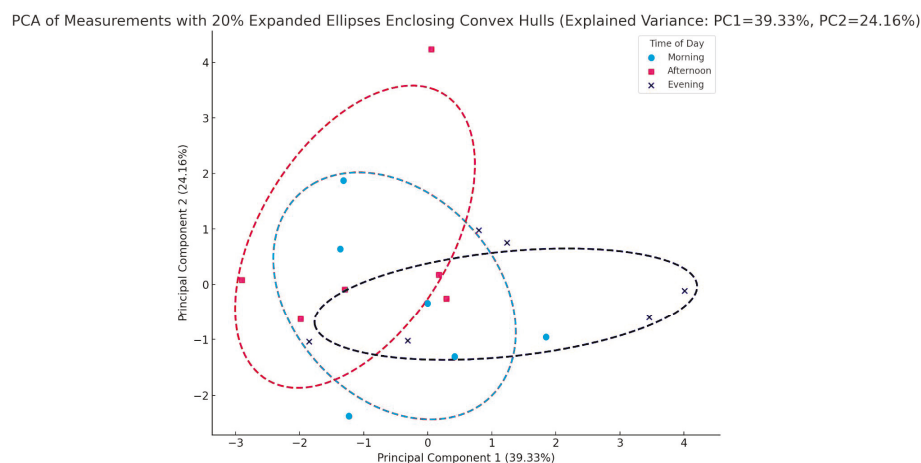
| Treatment                      | $\mu\text{m}$ | Thickness of the Adaxial Epidermis | Thickness of the Abaxial Epidermis | Thickness of the Palisade Parenchyma | Total Leaf Thickness |
|--------------------------------|---------------|------------------------------------|------------------------------------|--------------------------------------|----------------------|
| <i>C. siliqua</i> control      |               | $43 \pm 7^a$                       | $27 \pm 6$                         | $84 \pm 12$                          | $282 \pm 15^a$       |
| <i>C. siliqua</i> cold-treated |               | $30 \pm 5^b$                       | $20 \pm 5$                         | $69 \pm 15$                          | $180 \pm 12^b$       |
| <i>C. siliqua</i> heat-treated |               | $38 \pm 3^{ab}$                    | $24 \pm 6$                         | $75 \pm 11$                          | $213 \pm 16^b$       |



**Figure 2.** Scanning electron micrographs of the upper (adaxial) and lower (abaxial) epidermis from the leaves of *Ceratonia siliqua* after the end of the experiment; (a,b) the control plants; (c,d) the cold-stressed plants; and (e,f) the heat-stressed plants.

Chlorophyll concentration (Chl-a and Chl-b) was measured for 48 h after the end of the experiment. The malondialdehyde (MDA) content, indicating the oxidative stress the plants experience, was also defined, as well as the concentration of the total phenolic content (TPC). The samples for these parameters were collected at 08:00, 14:00, and 20:00 for day 1 and at 08:00, 14:00, and 20:00 for the day 2; the first sample was collected immediately after the end of the experiment (see Section 4). These parameters were analyzed using Principal Component Analysis (PCA) to detect any grouping of the specimens. The first PCA was utilized to explore temporal patterns in the dataset; the samples were grouped according to morning, afternoon, and evening timeframes, allowing for a nuanced examination of how measurements shift over the course of the day. The first two principal components, which together account for a substantial proportion of the total variance,

provided a clear visual framework for examining these temporal patterns and revealed distinct separations among these time-based clusters (Figure 3). By plotting the data in the space of the first two principal components, the clusters corresponding to different times of day emerged naturally, with morning, afternoon, and evening samples displaying distinct spatial separations. To better represent the inherent variability within each temporal cluster, ellipses were drawn around the central distributions of the data points. These ellipses serve to highlight the range and density of measurements, capturing the intrinsic dispersion within each cluster.



**Figure 3.** PCA analysis of MDA, TPC, Chl-a, and Chl-b in three clusters: morning samples, afternoon samples, and evening samples.

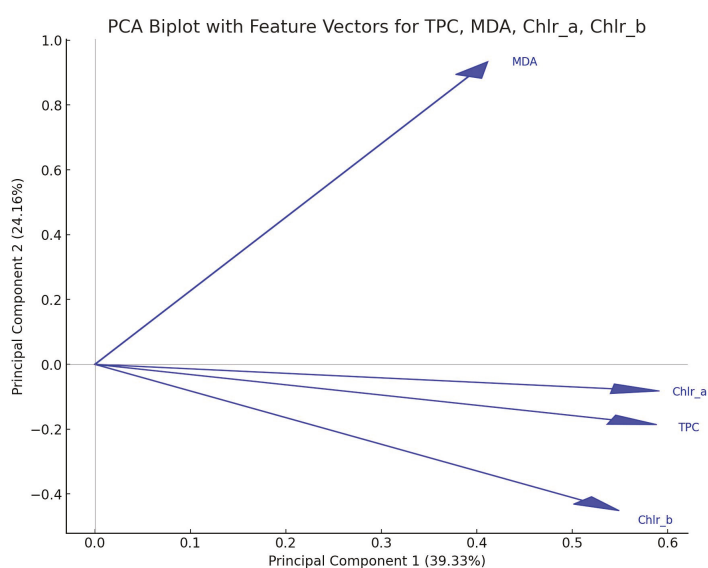
Notably, the morning cluster exhibited a more concentrated distribution along the first principal component, suggesting that measurements taken during this period were relatively uniform. In contrast, the afternoon and evening clusters spread more broadly across the principal component space, indicating greater variability in measurements later in the day. This pattern may reflect diurnal rhythms, shifts in environmental conditions, or other time-dependent biological and ecological factors that influence the measurements.

The second PCA analysis, a biplot, visualized the contributions of four features of stress physiology—TPC, MDA, Chl-a, and Chl-b—to the first two principal components (PC1 and PC2) derived from the dataset (Figure 4). Together, PC1 and PC2 account for a substantial share of the total variability (39.33% and 24.16%, respectively), offering a clear view of the dominant patterns within the data. In the biplot, each feature is represented by an arrow (or vector) radiating from the origin, with both the direction and length of the arrow providing crucial information about the feature's influence on the principal components. When a feature vector is closely aligned with one of the principal component axes, it indicates that the feature strongly contributes to the variance explained by that component. For example, TPC and MDA load heavily on PC1, suggesting that their variations are central to the primary dimension of variability in the data. Meanwhile, Chl-a and Chl-b exhibit contributions to both PC1 and PC2, implying that their variability plays a role in multiple axes of differentiation within the dataset. The spatial arrangement and relative positioning of these vectors not only shed light on how strongly each feature influences the principal components but also suggest correlations among the features themselves. Vectors pointing in similar directions, for instance, are often associated with positive correlations, whereas those pointing in opposite directions may indicate inverse relationships.

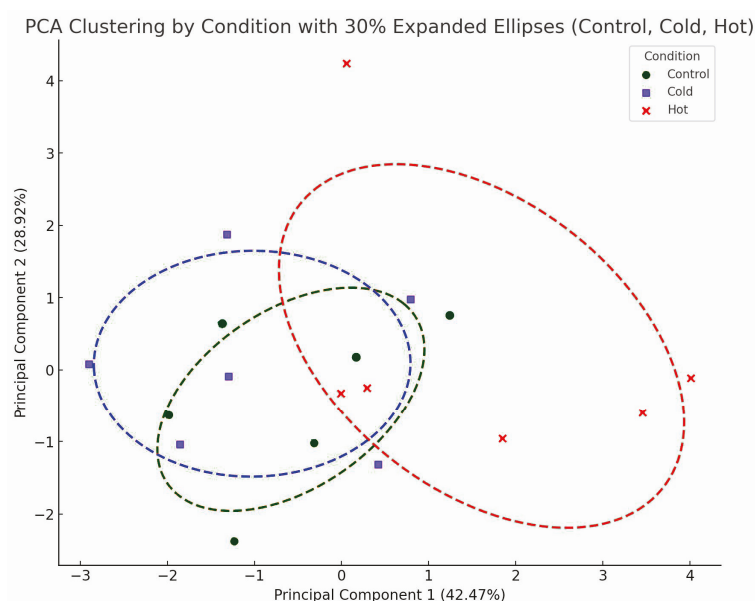
The last PCA plot illustrates the clustering of samples under three distinct conditions—control, cold, and hot—with ellipses to encompass the primary spread of data within each



group (Figure 5). Together, PC1 and PC2 explain a substantial portion of the dataset's total variance at 42.47% and 28.92%, respectively. By focusing on these two components, the plot provides a simplified yet informative view of the dominant patterns underlying the variability in the data. Each condition is represented by a distinct marker type (circles for control, rectangles for cold, and crosses for hot), with ellipses indicating the core distribution for each group. Ellipses were drawn around each cluster to indicate the core distribution of samples within that group, encapsulating most of the variability while mitigating the influence of potential outliers. These ellipses help to highlight the degree of overlap or separation among the three conditions. The apparent clustering and separation of the groups suggest that the treatments (in this case, temperature conditions) exert a measurable influence on the underlying variables captured by the PCA. These data indicate that severe heat stress has a major effect on carob physiology.



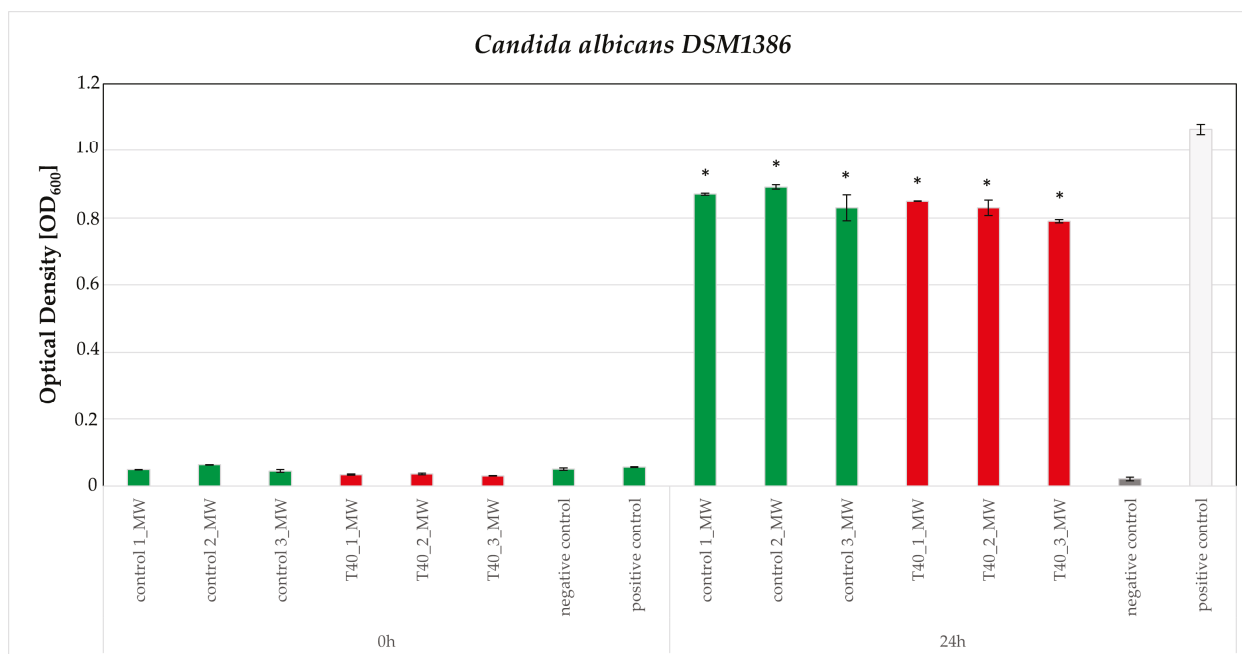
**Figure 4.** PCA biplot using data from TPC, MDA, Chl-a, and Chl-b measurements.



**Figure 5.** PCA analysis for TPC, MDA, Chl-a, and Chl-b measurements, clustered in three experimental conditions: control, cold, and hot.

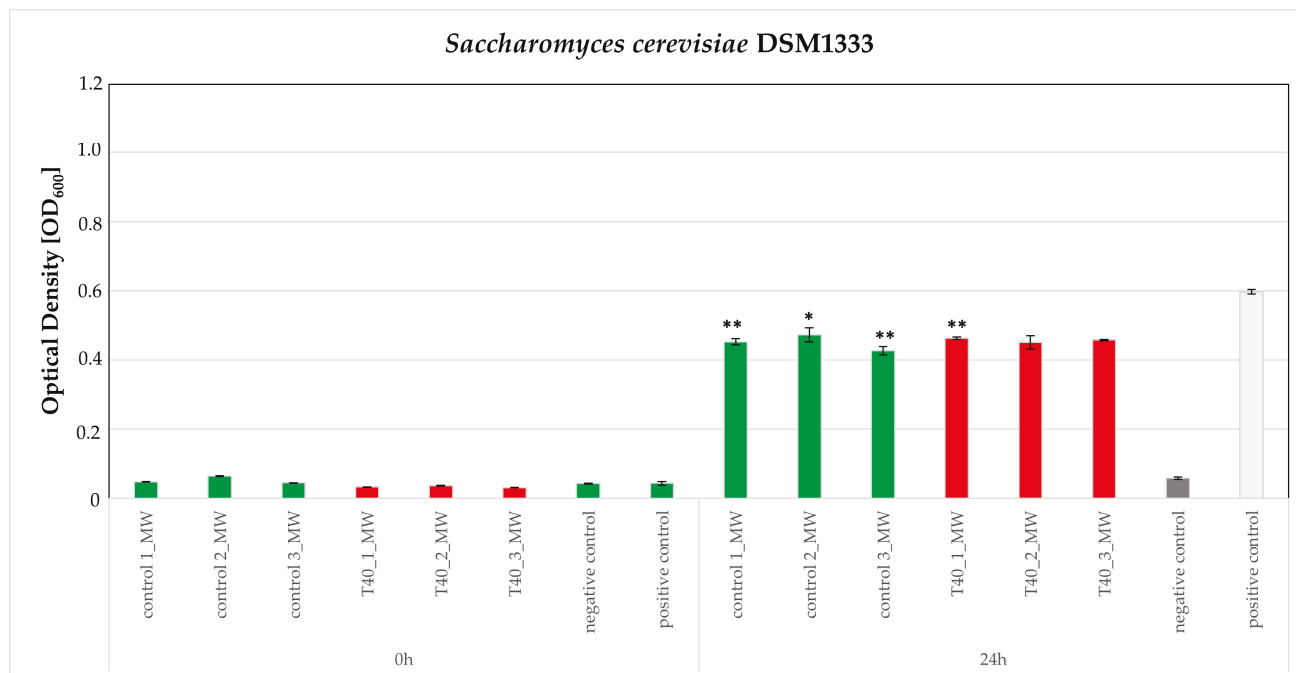
To gain a deeper understanding of the possible different composition of *C. siliqua* leaves following environmental stress, and as well as to examine the potential antimicrobial activity of the metabolites produced in the leaves of all treatments, both control and stressed leaves were extracted with the same method of using ultrasounds and increasing polarity solvents. Thus, the derived extracts, i.e., DCM (D), MeOH (M), and MeOH-H<sub>2</sub>O:50–50, v/v (MW) were first tested for their bioactivity on human pathogenic bacterial and fungal strains and specifically *Bacillus subtilis* DSM10, *Escherichia coli* DSM6897 *Pseudomonas aeruginosa* DSM50071, *Staphylococcus aureus* DSM346, *Candida albicans* DSM1386 and *Saccharomyces cerevisiae* DSM1333, and then their bioactivity against phytopathogenic microorganisms *Xanthomonas campestris* pv. *campestris* 1656 BPIC, *Pseudomonas syringae* pv. *syringae*, and *Erwinia amylovora* was also examined (see Section 4).

According to the susceptibility test of all nine microorganisms, only two human bacterial pathogens and one phytopathogenic bacterium were observed to have growth reduction, caused by the two of the three extracts, i.e., MW and D, respectively. This indicates that polar constituents abundant in MW exhibit an inhibitory effect against human pathogens while non-polar compounds, the components of D extract, are effective against phytopathogens. Furthermore, the stress conditions seem to affect extract potency, signifying qualitative and or/quantitative alternations of their composition. Specifically, control-MW and T40-MW extracts were found to reduce the growth of the human pathogens *C. albicans* DSM1386 and *S. cerevisiae* DSM1333 while T5-MW was found to be inactive. The observed growth reduction was found to be statistically significant compared to the positive control. However, when comparing the growth reduction effects caused by the control and T40-MW extracts, no statistically significant difference was observed (Figures 6 and 7).



**Figure 6.** Susceptibility testing of human pathogen *Candida albicans* DSM1386 based on growth in the presence of 200 µg/mL of the control-MW and T40-MW T40 extracts. (Positive control: a culture of the microorganism without added extract; negative control, a culture of the microorganism in the presence of Dimethylsulfoxide—DMSO—10%). The effect is evaluated in OD<sub>600</sub> measured at 0 and 24 h after inoculation. Estimated cutoff values and their standard errors for three independent experiments of triplicate datasets are plotted here. Asterisks (\*) represent statistically different data points at  $p \leq 0.05$ , according to *t*-test comparisons. (Green bars for the control and red for the heat-treated).

In contrast, control-D, T40-D, and T5-D extracts were found to reduce statistically significantly the growth of the phytopathogen bacterium *Erwinia amylovora* 842 BPIC, compared to the control. The observed growth reduction compared to the positive control (culture of the microorganism without added extract) is statistically significant according to the *t*-test analysis. Moreover, comparing the growth reduction effects caused by the control, T40, and T5 extracts, no statistically significant difference was observed (Figure 8). The other bacterial pathogens were fully grown, with no statistical significance compared to the positive control, when cultured in the presence of the extracts.

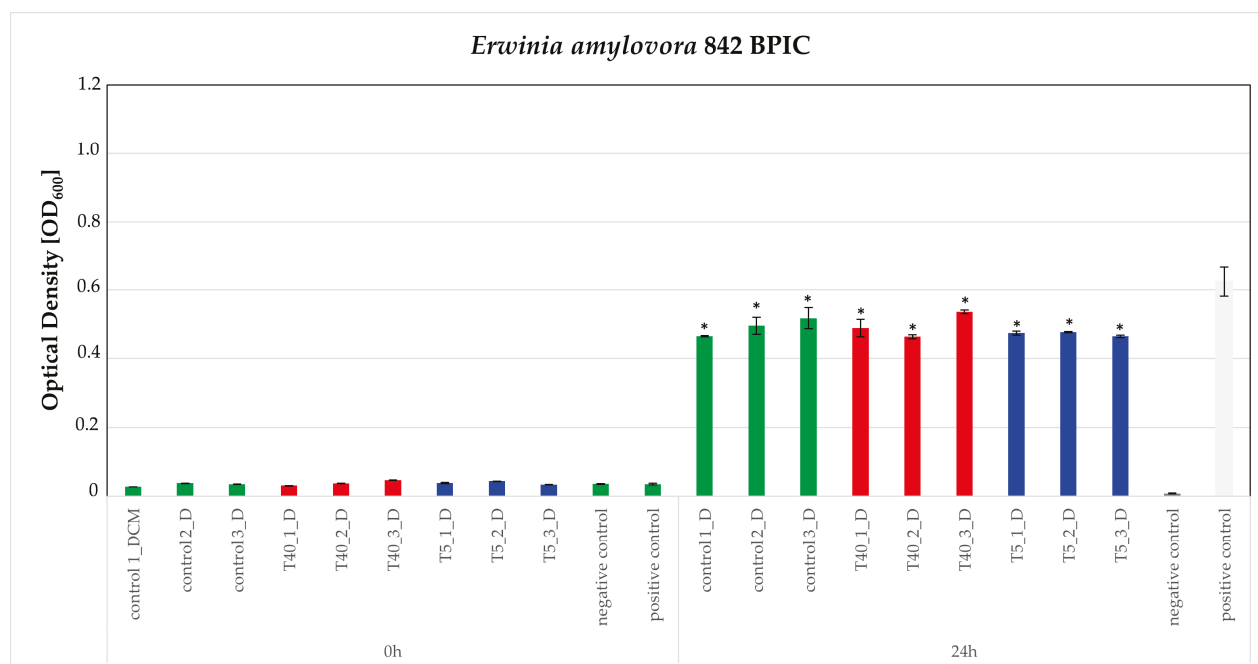


**Figure 7.** Susceptibility testing of the human opportunistic pathogen *Saccharomyces cerevisiae* DSM1333 based on growth in the presence of 200 µg/mL of control-MW and T40-MW extracts. (Positive control: a culture of the microorganism without added extract; negative control, a culture of the microorganism in the presence of DMSO 10%). The effect is evaluated in OD<sub>600</sub> measured at 0 and 24 h after inoculation. Estimated cutoff values and their standard errors for three independent experiments of triplicate datasets are plotted here. Asterisks (\*) Represent statistically different data points at  $p \leq 0.05$ ; Asterisks (\*\*) Represent statistically different data points at  $p \leq 0.01$ , according to *t*-test comparisons. (Green bars for the control and red for the heat-treated).

The obtained results from the susceptibility test on pathogens oriented the analysis and characterization of the most promising extracts. Thus, MW and D extracts were further investigated. Regarding D extracts, further sample treatment was necessary due to the chemical nature of the components (non-polar). To that end, the D extract was fractionated using LLE to two sub-extracts with the aid of EtOAc and n-Hexane as solvents. The first sub-extract with EtOAc (E extract) is compatible with LC-MS analysis (non-volatile compounds) and the second with n-Hexane (nH) is compatible with GC-MS analysis (volatile compounds). It is important to note that the use of two analytical platforms was necessary to compensate for analytical compatibility aspects originating from the samples' solubility and polarity [30]. In parallel, the initial M and MW extracts were also analyzed using LC-HRMS/MS to get better insight into the chemical composition of *C. siliqua* leaves.

In specific, suspect analysis was employed to dereplicate known compounds in the different extracts. Moreover, a comparative study between different extracts and stress conditions was undertaken under the metabolite profiling concept aiming to disclose

differences and similarities as well as marker compounds associated with stress. In total, 26 compounds were tentatively identified in all samples under investigation (M, MW, and E) using LC-HRMS/MS. The chromatographic and spectrometric data of the identified compounds in *C. siliqua* leaf extracts are presented in Table 3. According to our results, the M and MW extracts were rather similar in composition and mainly consisted of tannins, flavonoid glycosides, benzoic acids, and fatty alcohols, while the E extracts consisted of triterpenoids, methoxyphenols, benzofurans, medium-chain and long-chain fatty acids and glycerophosphoglycerols.



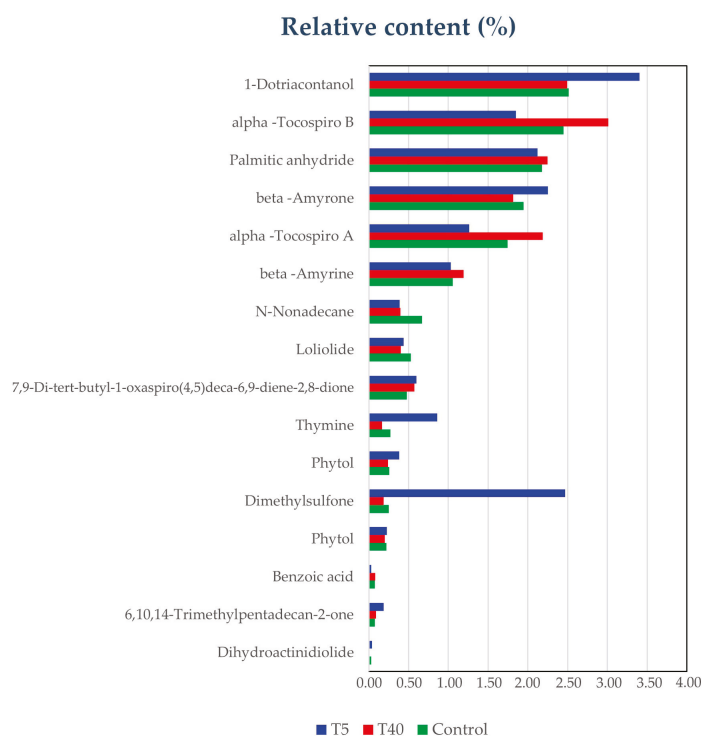
**Figure 8.** Susceptibility testing of phytopathogen bacterium *Erwinia amylovora* 842 BPIC based on growth in the presence of 200 µg/mL of the control-D, T40-D, and T5-D extracts. (Positive control: a culture of the microorganism without added extract; negative control, a culture of the microorganism in the presence of DMSO 10%). The effect is evaluated in OD<sub>600</sub> measured at 0 and 24 h after inoculation using a multi-detection microplate reader. Estimated cutoff values and their standard errors for three independent experiments of triplicate datasets are plotted here. Asterisk (\*) represents statistically different data points at  $p \leq 0.05$ , according to *t*-test comparisons (Green bars for the control, blue for the cold-treated and red for the heat-treated).

In more detail, the disaccharide maltose (1) and thymine (2) were the first two eluted compounds (0.88 and 0.89 min, respectively). Close in physicochemical properties were the compounds corresponding to peaks 3, 5, 6, 7, 10, 11, and 12, all belonging to the class of metabolites known as tannins (Table 3). These naturally occurring polyphenols can be divided into four primary classes: hydrolyzable tannins (based on ellagic acid or gallic acid), condensed tannins (made of oligomeric or polymeric proanthocyanidins), complex tannins (made of a catechin bound to a gallotannin or elagitannin), and phlorotannins (oligomers of phloroglucinol). Therefore, they were identified as di-galloyl-glucose (three isomers, eluting at 0.94 (3), 1.29 (6), and 1.60 min (7), respectively), glucogallin (5, 1.22 min), tri-galloyl-glucose (10, 4.34 min), and tetra-galloyl-glucose (12, 5.57 min). Peak 11 (5.07 min) was identified as epigallocatechin gallate, a primary metabolite containing a gallate moiety glycosidically linked to a catechin. Epigallocatechin gallate is a typical flavone-3-ol phenolic compound with eight free hydroxyl groups responsible for various biological functions [31].

Furthermore, the flavonoid-3-*O*-glycosides myricitrin (13, 5.73 min) and quercitrin (14, 6.22 min) were detected. HRMS/MS spectra contributed significantly to their identification with the most characteristic pattern the loss of the glucose unit and the detection of the aglycone. Regarding the rest of the chemical classes, they all comprise medium-chain and long-chain fatty acids, fatty amides, fatty acyl glycosides, and linolic acid derivatives. More specifically, after examining the HRMS/MS spectra the detected metabolites were azelaic acid (15, 6.42 min), 5,8,12-Trihydroxy-9-octadecenoic acid (17, 8.39 min), pipericine (18, 8.44 min), LysoPG (19, 16:0/0:0) (8.78 min), 13(*S*)-Hydroperoxylinolenic acid (20, 8.97 min), octadecenedioic acid (23, 10.34 min). Moreover, gingerol (16, 6.58 min) and two gingerol derivatives were detected, [6]-gingerdiol D-glucopyranoside (21, 9.12 min) and [12]-gingerdiol (24, 11.24 min), as well as two triterpenoids pomolic acid (25, 11.62 min) and ganoderol b (26, 12.32 min).

Regarding the nH sub-extract (fraction of bioactive D extract) and the GC-MS analysis, a total of 21 constituents were tentatively identified, accounting for 76.23% of the total peaks recorded. The stress conditions seem to have a stronger influence on the volatile-nonpolar constituents in comparison to polar ones, as indicated by the GC-MS chromatograms. Among these, glycerolipids played a major role with glycerol 1-palmitate being the most prominent, followed by long-chain fatty alcohols (2-stearoylglycerol). Other minor compounds that were identified were the triterpenoids germanicol, olean-18-ene, beta-amyrone, and beta-amyrine, as well as the lactone (7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione) and the benzofurans loliolide and dihydroactinidiolide. Additionally, the GC-MS analysis indicated the presence of  $\alpha$ -tocospiro A and  $\alpha$ -tocospiro B possessing unique 7,8-dimethyl-1-oxaspiro [4.4]-non-7-ene-6,9-dione carbon skeleton.

As illustrated in the bar graph of Figure 9, certain compounds seem to be altered by low or high temperatures. Interestingly, the two  $\alpha$ -tocopheroids ( $\alpha$ -tocospiro A and B), belonging to the general group of tocopherols were found to increase in high temperatures (T40).



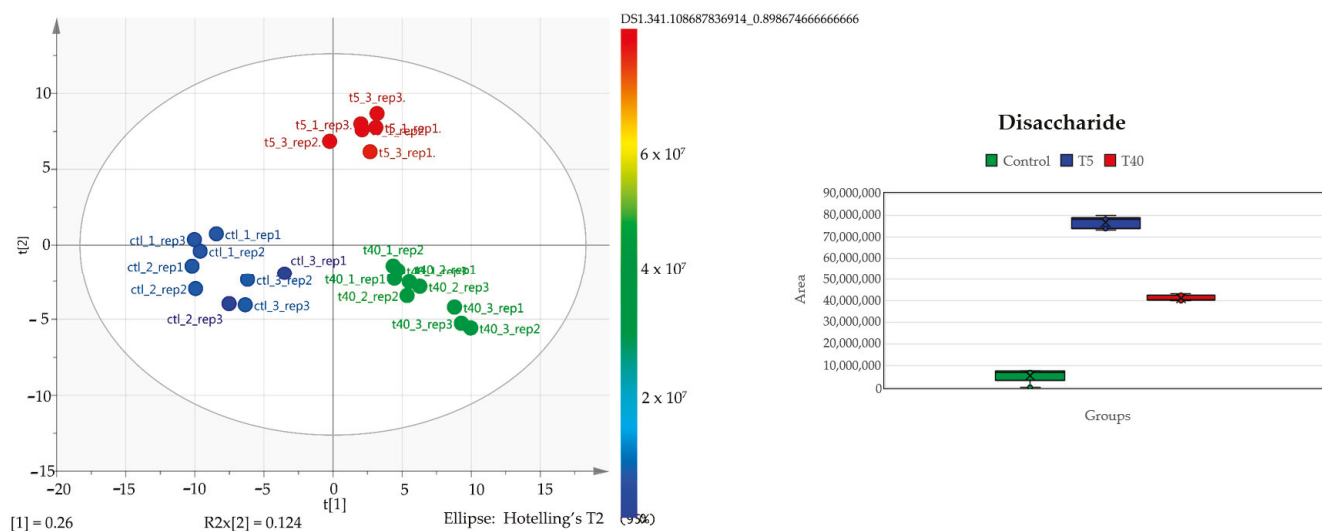
**Figure 9.** The bar graph of the nH extracts based on the percentage contribution (% peak area) of the metabolites identified between the different stress conditions using GC-MS analysis.

**Table 3.** UHPLC-Orbitrap-MS data of *C. siligua* L. leaves extracts identified compounds. RT: Retention time expressed in minutes; exp. m/z: Experimental m/z of pseudomolecular ion; Theoretical m/z;  $\Delta m$ : mass accuracy expressed in ppm. RDBeq.: Ring and double bond equivalent; Spectrometric information for characteristic HRMS/MS fragment ions are also given.

| ID | Mode | Compound                                                                   | RT (min)   | Exp. m/z  | Theor. m/z | $\Delta m$ (ppm) | Formula                                                     | RDB  | Fragment Mass                       | Chemical Class                            |
|----|------|----------------------------------------------------------------------------|------------|-----------|------------|------------------|-------------------------------------------------------------|------|-------------------------------------|-------------------------------------------|
| 1  | Neg  | Disaccharide (Maltose)                                                     | 0.88       | 341.10904 | 341.1089   | 0.4              | C <sub>12</sub> H <sub>22</sub> O <sub>11</sub>             | 2.5  | 179.0562/161.0456/281.0885          | Carbohydrates and carbohydrate conjugates |
| 2  | Pos  | Thymine                                                                    | 0.89       | 127.0502  | 127.0502   | 0.0              | C <sub>5</sub> H <sub>6</sub> N <sub>2</sub> O <sub>2</sub> | 3.5  | 84.0445/109.0399                    | Pyrimidines and pyrimidine derivatives    |
| 3  | Neg  | Di-galloyl-glucose (isomer 1)                                              | 0.94       | 483.0771  | 483.0769   | 0.4              | C <sub>20</sub> H <sub>19</sub> O <sub>14</sub>             | 11.5 | 331.0667/313.0562/169.0140          | Tannins                                   |
| 4  | Pos  | Loliolide                                                                  | 1.13       | 197.1172  | 197.1172   | 0.0              | C <sub>11</sub> H <sub>16</sub> O <sub>3</sub>              | 3.5  | 179.1068/135.1169/161.0962          | Benzofurans                               |
| 5  | Neg  | Glucogallin                                                                | 1.22       | 331.0664  | 331.066    | 1.2              | C <sub>13</sub> H <sub>15</sub> O <sub>10</sub>             | 6.5  | 271.0456/211.0245/169.0140          | Tannins                                   |
| 6  | Neg  | Di-galloyl-glucose (isomer 2)                                              | 1.29       | 483.0771  | 483.0769   | 0.4              | C <sub>20</sub> H <sub>19</sub> O <sub>14</sub>             | 11.5 | 331.0667/313.0562/169.0140          | Tannins                                   |
| 7  | Neg  | Di-galloyl-glucose (isomer 3)                                              | 1.60       | 483.0771  | 483.0769   | 0.4              | C <sub>20</sub> H <sub>19</sub> O <sub>14</sub>             | 11.5 | 331.0667/313.0562/169.0140          | Tannins                                   |
| 8  | Neg  | 6-O-(hydroxybenzoyl)hexopyranosyl<br>6-O-(trihydroxybenzoyl)hexopyranoside | 2.5        | 443.1191  | 443.1184   | 1.6              | C <sub>19</sub> H <sub>23</sub> O <sub>12</sub>             | 8.5  | 331.0669/313.0564/193.0141          | Benzoic acids and derivatives             |
| 9  | Neg  | Methyl gallate                                                             | 4.23       | 183.0296  | 183.0288   | 4.4              | C <sub>8</sub> H <sub>7</sub> O <sub>5</sub>                | 5.5  | x                                   | Benzoic acids and derivatives             |
| 10 | Neg  | Tri-galloyl-glucose                                                        | 4.35/5.04  | 635.0877  | 635.0879   | −0.3             | C <sub>27</sub> H <sub>23</sub> O <sub>18</sub>             | 16.5 | 465.0668/421.0773                   | Tannins                                   |
| 11 | Neg  | epigallocatechin gallate                                                   | 5.07       | 457.0742  | 457.0765   | 2.0              | C <sub>22</sub> H <sub>17</sub> O <sub>11</sub>             | 14.5 | x                                   | Flavans (catechin gallates)               |
| 12 | Neg  | Tetra-galloyl-glucose                                                      | 5.57       | 787.0984  | 787.0988   | −0.5             | C <sub>34</sub> H <sub>27</sub> O <sub>22</sub>             | 21.5 | 635.0887/617.0782/465.0676          | Tannins                                   |
| 13 | Neg  | Myricitrin                                                                 | 5.73       | 463.0877  | 463.0871   | 1.3              | C <sub>21</sub> H <sub>19</sub> O <sub>12</sub>             | 12.5 | 317.0298/316.0221                   | Flavonoid glycosides                      |
| 14 | Neg  | Quercitrin                                                                 | 6.22       | 447.0928  | 447.0922   | 1.3              | C <sub>21</sub> H <sub>19</sub> O <sub>11</sub>             | 12.5 | 301.0352                            | Flavonoid glycosides                      |
| 15 | Neg  | Azelaic acid                                                               | 6.42       | 187.0976  | 187.0976   | 0.2              | C <sub>9</sub> H <sub>16</sub> O <sub>4</sub>               | 2.5  | 125.0971/143.1077/169.0871          | Medium-chain fatty acids                  |
| 16 | Pos  | Gingerol                                                                   | 6.58/10.09 | 293.1758  | 293.1758   | 0.0              | C <sub>17</sub> H <sub>26</sub> O <sub>4</sub>              | 5.5  | 221.1547                            | Methoxyphenols                            |
| 17 | Neg  | 5,8,12-Trihydroxy-9-octadecenoic acid                                      | 8.39       | 329.2333  | 329.2333   | −0.1             | C <sub>18</sub> H <sub>34</sub> O <sub>5</sub>              | 2.5  | 311.2231/171.1027                   | Long-chain fatty acids                    |
| 18 | Pos  | Piperidine                                                                 | 8.44       | 336.3260  | 336.3261   | −0.3             | C <sub>22</sub> H <sub>41</sub> NO                          | 2.5  | 319.3001/301.2896                   | Fatty amides                              |
| 19 | Pos  | LysoPG(16:0/0:0)                                                           | 8.78       | 485.2885  | 485.2874   | 2.3              | C <sub>22</sub> H <sub>45</sub> O <sub>3</sub> P            | 0.5  | 351.1803/467.2787                   | Glycerophosphoglycerols                   |
| 20 | Neg  | 13(S)-Hydroperoxylinolenic acid                                            | 8.97       | 309.2071  | 309.2071   | 0.0              | C <sub>18</sub> H <sub>30</sub> O <sub>4</sub>              | 4.5  | 291.1967/239.1655/183.1029/273.1864 | Lineolic acids and derivatives            |
| 21 | Neg  | [6]-Gingerdiol D-glucopyranoside                                           | 9.12       | 457.2442  | 457.2443   | −0.2             | C <sub>23</sub> H <sub>38</sub> O <sub>9</sub>              | 5.5  | 181.0719                            | Fatty acyl glycosides                     |
| 22 | Pos  | MG(16:0/0:0/0:0)                                                           | 10.18      | 313.2734  | 313.2743   | −2.9             | C <sub>19</sub> H <sub>38</sub> O <sub>4</sub>              | 1.5  | 257.2477/239.2371                   | Monoradylglycerols                        |
| 23 | Neg  | Octadecenedioic acid                                                       | 10.34      | 311.2227  | 311.2228   | −0.3             | C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>              | 3.5  | 293.2124                            | Long-chain fatty acids                    |
| 24 | Pos  | [12]-Gingerdiol                                                            | 11.24      | 381.2995  | 381.2999   | −1.0             | C <sub>23</sub> H <sub>40</sub> O <sub>4</sub>              | 3.5  | 265.2556/165.1638/135.1169/121.1012 | Methoxyphenols                            |
| 25 | Neg  | Pomolic acid                                                               | 11.62      | 471.3483  | 471.3480   | 0.6              | C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>              | 7.5  | 425.3427/339.2694                   | Triterpenoids                             |
| 26 | Pos  | Ganoderol B                                                                | 12.32      | 423.3613  | 423.3627   | −3.3             | C <sub>30</sub> H <sub>48</sub> O <sub>2</sub>              | 7.5  | 405.3515/189.1638/283.2421          | Triterpenoids                             |

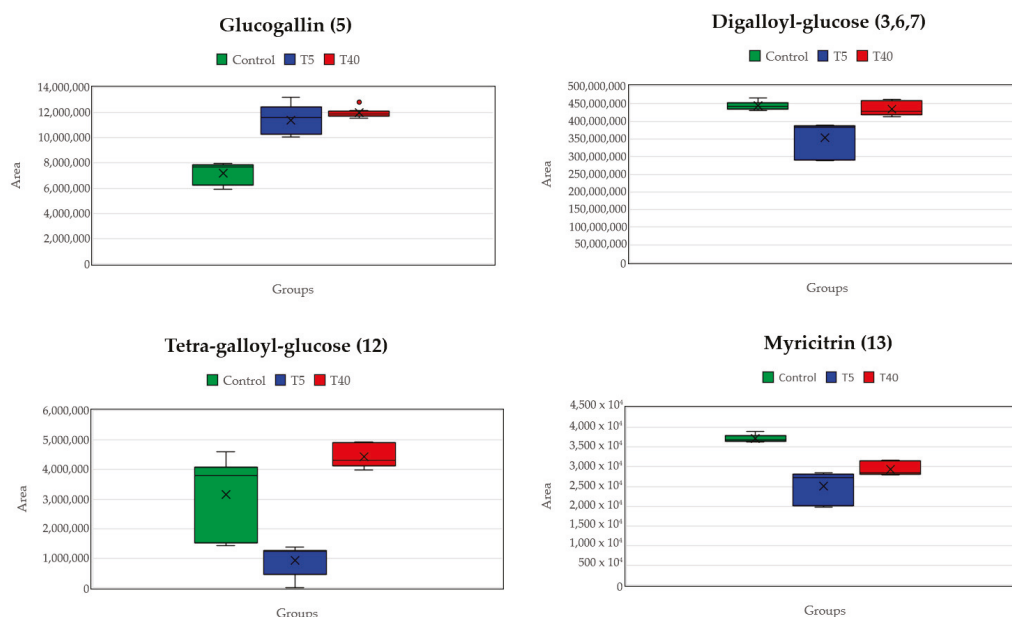


As mentioned, apart from suspect analysis and dereplication procedure, the LC-MS/MS data were subjected to metabolite profiling to disclose differences between stress conditions and to reveal marker compounds. Multivariate analysis (MVA) was used for descriptive analysis and investigation of trends and classifications based on the secondary metabolites (Figure 10).



**Figure 10.** (Left): PCA; scatter plot scores of MW extracts. Control leaves samples (blue) and stressed leaves samples [T40 (green) and T5 (red)] are presented; (Right): Boxplot indicating the variation of concentration levels of the detected disaccharide 1 amongst the three sample groups.

It is obvious from the scores plot that the samples are clustered separately, forming three distinct groups. At the first principal component, control and high temperature (T40) are clearly separated, and at the second component, the group of low temperature (T5) is also separated from the other two groups. These trends indicate metabolism alternation under the different treatments. Proceeding with supervised methods and specifically OPLS-DA it was possible to identify statistically significant features which are up- or down-regulated under stress, in comparison with control samples. A notable feature is the elevated levels of a disaccharide, tentatively identified as maltose (1; Table 3), observed in both low- and high-temperature samples compared to the control. From the OPLS-DA plot, it appears that low temperatures have a more pronounced effect. In Figure 11, the metabolites exhibiting the most significant variation due to temperature changes are presented; as illustrated, heat stress conditions alter the hydrolyzable tannin levels. Specifically, high temperatures seem to promote the up-regulation of hydrolyzable tannins, while low temperatures result in reduced levels. Furthermore, the number of esterified gallic acid units on the glucose scaffold might also play a role. It is noteworthy that not all metabolites respond in the same manner when the temperature increases or decreases. Regarding flavonol glucosides, myricitrin, exhibits a reduction at both low and high temperatures, whereas glucogallin, in contrast, increases under cold and hot stress. Furthermore, tetra-galloyl-glucose displays a linear relationship with temperature, meaning that as the temperature rises, its levels also increase, and as the temperature drops, so do its levels. Meanwhile, digalloyl-glucose remains stable at 40 °C but decreases noticeably in colder conditions. These temperature-dependent fluctuations of metabolite concentrations indicate that the plants respond to environmental changes in multiple ways that need further investigation.



**Figure 11.** Indicative boxplots of detected compounds presenting statistically significant altered concentration levels under different stress conditions.

### 3. Discussion

Climate change results in the exposure of plants and animals to an abiotic environmental stress to which they are not familiar. Drought, warmer long-lasting summers, and short yet chilling winters, as well as an increase in the global temperature over the years, force all organisms to adapt to the new conditions. Stressing conditions restrict plants' development and thus their survival fate. In our experiments, carob tree plants chilled for only six days produced less biomass than their control counterparts while the heat-stressed individuals were far more retarded in their final yield. It seems that, although *C. siliqua* is a cold-sensitive species, the high temperatures prove to be far more distressing.

#### 3.1. Leaf Structure

It seems that the plants exposed to the chill stress appear less xeromorphic. The plants exposed to heat stress reacted by producing compact leaf tissues with their cells accumulating phenolics; it is known that drought stress results in a decrease in leaf size [32]. The leaves of *C. siliqua* did not seem to exaggerate any of their structural features, compared to some other Mediterranean evergreen sclerophylls [13]. They withstood the high temperatures accumulating phenolics, thus reducing their productivity, while their response to low temperatures was weak and most of the time did not avoid cell injury (Table 1).

The epidermal tissues, which are the thickest among the leaves of the Mediterranean evergreen sclerophylls [13] did not seem to be affected (Figure 2). GC-MS analysis demonstrated the production of  $\alpha$ -tocospiro A and  $\alpha$ -tocospiro B in heat-stressed samples, both being rare  $\alpha$ -tocopherol derivatives. It has been well documented that the levels of tocopherols as strong antioxidants, are related to stress tolerance in plants [33] towards the management of excess ROS levels. In contrast, 1-dotriacintanol (fatty alcohols), dimethylsulfone (sulfones), and beta-amyrone (triterpenoids) are up-regulated in cold conditions. All these different chemical classes have been related to the response mechanisms of plants to compensate for abiotic stress [34–36]. Tocopherol (Vitamin E) is an antioxidant molecule that is considered to be a major scavenger of lipid peroxyl radicals produced in thylakoid membranes to preserve their structure and function during stress conditions [37–39]. Fur-

thermore, tocopherol external application resulted in increased biomass in *Vicia faba* and *Hibiscus rosasineses* due to the enhanced antioxidant capacity of the plants [40–42]. In addition, maltose, a disaccharide, occurs in high concentration in cold- and heat-stressed samples when compared to control ones, and is recognized to stabilize the structure of chloroplast stroma in hot (40 °C) and cold stress (5 °C) conditions in *Arabidopsis thaliana* [43]. It has also the ability to protect proteins, membranes, and the photosynthetic electron transport chain at physiologically relevant concentrations under heat stress and freezing stress [43].

Regarding the flavonol glucoside myricitrin (13-Table 3), we monitored this and it was found in lower concentration in exposed samples compared to control in both conditions. While myricitrin is a metabolically or physiologically non-essential metabolite, it may serve as a defense or signaling molecule. This down-regulation relative to the control samples could be associated with metabolic changes triggered by thermal stress. The other flavonol, quercitrin (14-Table 3) breaks down to form quercetin and rhamnose. It is reported that quercetin reduces the level of H<sub>2</sub>O<sub>2</sub> (required for ABA-induced stomatal closure) that reduces the stomatal closure, which helps the plants face stress in a savior manner [44,45]. Closing of stomata is among the major reactions of Mediterranean plants against summer drought and cold stress [46]. In GC-MS analysis, glycerolipids played a major role with glycerol 1-palmitate being the most prominent, followed by long-chain fatty alcohols (2-stearoylglycerol). It is known that fatty acid production plays a fine-tuning role in drought stress, preserving the structure of cell membranes [47].

### 3.2. Chlorophyll, MDA, and TPC Content

Both chlorophyll-a and -b follow the same concentration pattern. The concentration declines until midday and starts to rise again, to reach the top, in late afternoon. MDA follows a concentration pattern different from that of chlorophyll absorbance (Figure 4). This stress indicator keeps rising during the day to reach the maximum in the late afternoon. This is true for the plants of all three groups. Furthermore, in Figure 3 it is obvious that in the morning, physiology parameters seem to have a more concentrated distribution, indicating the setup of a possible “reset” mechanism, zeroing all environmental affecting factors. The plant, after 6 and 12 h, must face up to environmental pressures leading to oxidative stress and the generation of secondary metabolism to create “safeguard” substances for their survival. Indeed, the plants exposed to high temperatures experience stressful conditions, their response being obvious from late noon to late afternoon. The cold-exposed plants seem to experience stressful conditions as well, yet their response, concerning the accumulation of oxidative factors, seems milder (Figure 5). This augmentation in the MDA concentration following heat stress is in accordance with ref. [48], where heat-stressed (32 °C) seedlings of *C. siliqua*, suffering drought stress, reported an increase in MDA content. Moreover, signs of lipid peroxidation damage were detected in heat-treated plants, in which drought caused an increase of 40% in malondialdehyde (MDA) content. Epigallocatechin gallate, a phenolic compound responsible for various biological functions [31], is synthesized via the flavonoid biosynthesis pathway. It is one of the most important secondary metabolites that can relieve plants from temperature stress. In tea plants, a 6-day exposure to 35 °C increased the concentration of epigallocatechin gallate, pointing out its role in the adaptation to high temperatures [49]. Exogenous induced epigallocatechin gallate relieved tea plants from heat stress, leading to augmentation of apoplastic ROS signaling; the same motif is true for tomato plants; exogenous epigallocatechin gallate resulted in apoplastic ROS production, offering a resistance to tobacco mosaic virus (TMV) [50]. Furthermore, epigallocatechin gallate also emerges as a safeguard metabolite for cold stress, increasing ROS and MDA levels in tea plants [51]. According to Keunen et al. [52] there is a strong relationship

between sugars and especially disaccharides with stress-induced ROS accumulation in plants. In our data, epigallocatechin gallate seems to remain in the same concentration in both treatments; this fact indicates that *C. siliqua* can withstand stress.

The TPC seems to follow the pattern of the chlorophyll-a and -b content in the plants of all three groups. Considerable differences in the diurnal concentration were observed in the plants of the heat-stressed group. This diurnal variation of phenolics means that not all of them are terminal metabolic products, i.e., tannins, but some of the phenolics are hydrolyzable and present a degree of mobility, probably of high importance for the plant. Phenolic compounds are classified into flavonoids and non-flavonoids [53]. Phenolic acids (e.g., gallic acid) function as precursor molecules of hydrolyzable tannins. Tannins and phenolic acid are non-flavonoids. Tannins are amongst the most characteristic classes of polyphenols that have been reported for their implication in plant stress tolerance [54]. Figure 5 PCA plot provides a clear visual of how each condition influences the overall data structure, suggesting condition-specific patterns. Heat stress seems to provoke a greater effect on plants' physiology, indicating that extreme prolonged temperature affects oxidative stress and the accumulation of phenolics. Heat stress conditions in *Festuca trachyphylla* favor the increased expression of hydrolyzable tannins and phenolic acids [55–57]. The accumulation of tannins is considered to be the factor that enhances plants' resistance after ROS scavenging [58]. This is in accordance with our data, in which MDA concentration and TPC appeared not to follow the same pattern but were time-delayed (Figure 4).

Given that the tissues of *C. siliqua* can survive in a narrow span of low temperatures, from above  $-6^{\circ}\text{C}$  for the leaves to above  $-11^{\circ}\text{C}$  for the stem xylem, compared to the other Mediterranean evergreen sclerophylls (Table 3) [59], we may assume that the plant has not invested in overcoming the chilling effects thus the response of the cold-stressed plants is rather inconspicuous.

### 3.3. Bioassays

It is important to note that MW extracts from the control and heat-stressed samples could inhibit the growth of *C. albicans* and *S. cerevisiae*, human pathogen strains. On the other hand, inhibition of the growth of phytopathogen Gram-negative bacterium *Erwinia amylovora*, following incubation with D extracts (control, T40, and T5) was also notable. The consequent incubation with the two sub-extracts of D (nH and E), did not exhibit any significant effect on the inhibition of *Erwinia amylovora* growth. That was not expected and could imply that the observed activity is the result of a synergistic effect commonly reported for complex mixtures such as plant extracts. Further deconvolution of this complexity is required to reveal the active compound and/or compounds [57,58], and *E. amylovora* is considered the “guilty” one for the fire blight disease on many individuals of the Rosaceae family [60]. Therefore, it is important that D extract from *C. siliqua* leaves, control or heat- and cold-stressed, could inhibit the growth of this bacterium and could have a drastic effect on relieving the plants that suffer from the “attack” of *E. amylovora*.

## 4. Materials and Methods

### 4.1. Plant Material and Exposure Setup

The seeds of *Ceratonia siliqua* are brown, with extremely tough, impermeable seed coats exhibiting external physical dormancy impeding water absorption [61], thus hindering germination [62,63]. They sprout, in nature, either from an entire pod buried in the soil or from seeds that had been consumed by certain bird species, digested, and eliminated far away. They also sprout, eventually, after wildfires, which are very frequent in the areas where this species occurs. In the laboratory, they do not sprout unless they

undergo a dormancy-breaking treatment. Pods of *C. siliqua* (Figure 12a) were collected from the island of Ios, Greece (36°44'02.8" N 25°16'37.1" E). They were crushed manually to extract the seeds. For the current experiment, acid scarification was used. Seeds of *C. siliqua* were immersed in 65% sulphuric acid for 10 min [64,65]. The treated seeds were washed for 30 min with running tap water, dried, and drenched with fungicidal powder [Thiram; (Tetramethylthiuram disulfide)—IUPAC name: dimethylcarbamoithioylsulfanyl N,N-dimethylcarbomodithioate—U244 EPA code, Sigma-Aldrich, St. Louis, MO, USA]. One hundred seeds were imbibed in three Petri dishes, each one containing two sheets of filtered paper, and grown at 22°C (70% humidity), in a light/dark cycle of 16 h/8 h and 110  $\mu\text{mol}/\text{m}^2/\text{s}$  of (PAR) illumination supplied by cool-white, fluorescent tungsten tubes (Osram, Germany). The germination procedure was completed in 5 days, scoring almost 99% (98 seeds out of 100 were germinated; 1 was empty). Germinated seeds (radicle protrusion about 1 cm) were sown in 30 × 10 × 10 cm, 3.0 L PVC long pots trays filled with wet Potgrond P medium (Klasmann–Deilmann), at pH 6.0 (Figure 12b), with 10 seedlings in each pot tray. Initially, two pot trays with 10 seedlings each were placed to grow in each one of the three culture chambers. To ensure the possibility of replacing any loss during the 6-week adaptation period and prior to the application of the stress conditions, an additional pot tray with 10 seedlings as well was added to the “control” culture chamber; however, after all, there was no need for using any additional seedlings. Two pot trays (tray 1 and tray 2) were incubated in each one of the temperature-controlled plant growth culture chambers (elvem—model BOD100, Spata 19004, Athens, Greece).



**Figure 12.** (a) The leaves and the pods containing the seeds of a wild growing tree of *Ceratonia siliqua*; (b) The pots with the carob tree plantlets at the beginning of the experiment.

The culture chambers have built-in illumination sources: two 1000 lumen, 10 W LED at 4500 K assisted by two Osram basic, T5 Short, L 6 W/640 miniature tubes, length 212 mm, diameter 16 mm, 4000 K. Total illuminance at the surface of the pots, directly measured with a MASTECH MS6610 portable luxmeter (MASTECH Group, Taipei, Taiwan), was 125.7 to 128  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Figure 13a–c).

Air was supplied through a HAILEA ACO-9160 (Chaozhou, China) air pump, at an output of 2 L/min for each incubator. All incubators were initially set at 25.0 °C ( $\pm 0.2$ ), a temperature reported to be appropriate for the germination and growth of *C. siliqua* [55,59]. The incubator temperature was constantly monitored with an industrial, two-channel data logging (Model AZ9882, AZ Instrument Crop., Taichung, Taiwan) thermometer (probe sensitivity: approximately 43  $\mu\text{V}/^\circ\text{C}$ ) through the attached Type T (copper–constantan) needle-shaped thermocouples.

To ensure that all plants in each incubator received the same light energy, we changed the position of each pot tray at 09:00 every morning, so that the pot trays alternated from being, each, either at the left, in the middle, or at the right side within the incubator. The seedlings of all three groups were left to grow for six weeks at a 24 h cycle of 25 °C (adaptation period). Then, the 6-day temperature stress period followed. The temperature

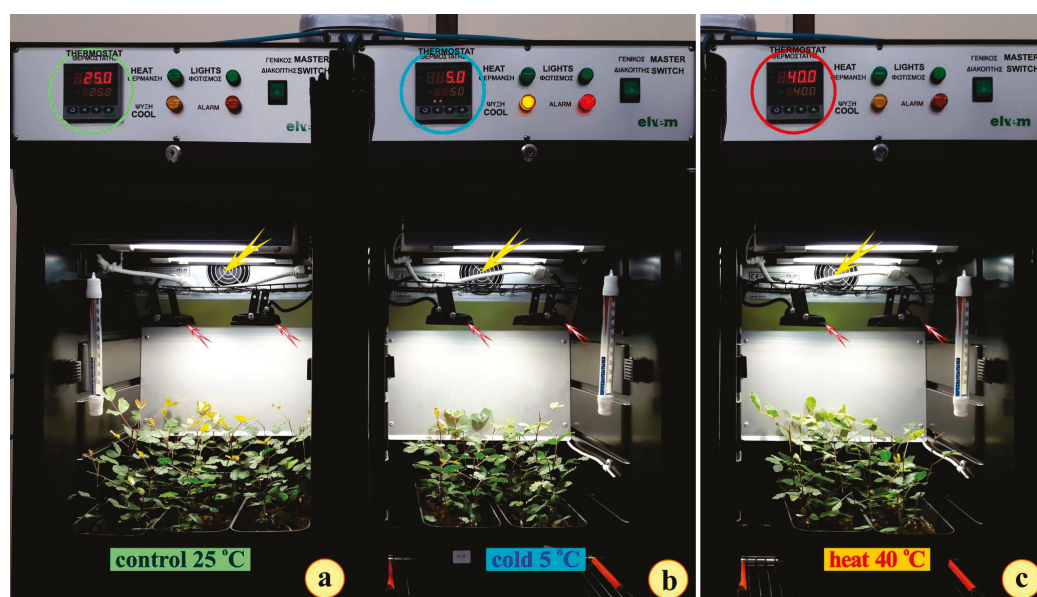


in each incubator was adjusted as follows: 25.0 °C (24 h cycle) for the control plants (T25) (Figure 13a); 5.0 °C (24 h cycle) for the cold-stressed plants (T5) (Figure 13b) and 40.0 °C (24 h cycle) for the heat-stressed plants (T40) (Figure 13c). The pots in each of the three chambers received 110  $\mu\text{mol}/\text{m}^2/\text{s}$  of (PAR) illumination for a light/dark cycle of 16 h/8 h, for six days and nights. At the end of this period, sampling for chlorophylls, total phenolic content, and MDA estimation were performed as follows: (1) by the end of the experiment (08:00), (2) at 14:00, (3) at 20:00, and for the next day (4) at (08:00), (5) at 14:00, and (6) at 20:00.

#### 4.2. Microscopy

At the end of the experiment, the pot trays were removed from the three culture chambers and their contents were dispersed in water to remove the culturing substrate from the roots of the plants.

Small parts, adjacent to the central nerve, were removed at random from the middle of the younger, upper pair leaves on each plant, cut into small pieces ( $1 \times 1 \text{ mm}$ ), and fixed in phosphate-buffered 3% glutaraldehyde (pH 6.8) at 0 °C for 2 h. The tissue was post-fixed in 1% osmium tetroxide in phosphate buffer and dehydrated in graded ethanol series. The properly prepared pieces were either (a) transferred in 100% acetone, critical point dried, coated with gold, and viewed with a JEOL JSM-6360 Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan), or (b) embedded in Durcupan ACM (Fluka, Steinheim, Switzerland).



**Figure 13.** The experimental setup and the plants of *Ceratonia siliqua* at the end of the experiment; (a) the plant growth culture chamber with the two pot trays of the control plants and the additional spare pot tray. The temperature was adjusted to 25.0 °C (T25 plants) (green cycle); (b) The culture chamber with the cold-stressed plants. The temperature adjusted to 5.0 °C (T5 plants) (cyan cycle); (c) The culture chamber with the heat-stressed plants. The temperature adjusted to 40.0 °C (T40 plants) (red cycle). The small white arrows point at the 10 W, 1000 lumen LED floodlights; the yellow arrows point at the temperature-stabilizing fan.

Stomatal frequency was measured on the peeled abaxial epidermis of 5 mature leaves, in 30 different optical fields for each leaf. Average stomatal frequency as well as standard deviation were calculated. Statistical analysis using the Tukey test was performed for the average stomatal frequency.



Semithin sections (5–6 microns) obtained from an LKB Ultratome III (Stockholm-Bromma 1, Sweden), were placed on glass slides, and stained with 0.5 toluidine blue O (in 1% borax solution), as a general stain, for light microscopic observations. Ultrathin sections (50–60 nm) were placed on 100 mesh grids, double stained with uranyl acetate and lead citrate [66], and viewed with a Phillips EM-300 Transmission Electron Microscope (Phillips, Amsterdam, The Netherlands). The literature on double fixation is cited in detail [67,68].

#### 4.3. Pigments Protocol

Total chlorophyll-a and -b (Chl-a and Chl-b) content was spectrophotometrically determined. Approximately 50 mg fresh leaves, 10 leaf samples from each treatment, were extracted with 1 mL 80% (v/v) acetone, overnight, at 4 °C. Supernatant was transferred to a 1.5-mL glass cuvette for measurement in v-1200 spectrophotometer (WVR; Pennsylvania, PA, USA). Absorbance was read at both 663.6 and 646.6 nm, corresponding to chlorophyll-a and chlorophyll-b respectively. Quantification of pigment content was calculated using molar extinction coefficients specifically for this method and normalized per fresh weight (mg/g) [69]. Chlorophyll-a,  $e_{663.6} = 76.79$  and  $e_{646.6} = 18.58$ ; chlorophyll-b,  $e_{663.6} = 9.79$  and  $e_{646.6} = 47.04$  [70]. All data was expressed as the mean of ten samples  $\pm$  standard error of the mean.

#### 4.4. Protocol for MDA (Malondialdehyde) Determination in Plant Tissues

Extraction of MDA from 50 mg plant material (frozen and ground in liquid nitrogen) was executed using 1 mL 0.25% thiobarbituric acid (TBA—Sigma-Aldrich™, Milan, Italy) dissolved in 10% trichloroacetic acid (TCA—Sigma-Aldrich™, Milan, Italy). The extract was collected in a 1.5-mL Eppendorf tube, and the mixture was heated at 85 °C for 30 min, and then quickly chilled on ice. The same procedure was followed for the blank sample but without plant material. The mixture was centrifuged at maximum speed for 10 min to pellet the particles. The supernatant was then transferred in a 1-mL plastic cuvette for spectrophotometric measurement. The absorbance was determined primarily at 532 nm (the peak of the MDA–TBA complex) and then at 600 nm (nonspecific absorption). 1 mL 0.25% TBA in 10% TCA was used as a blank. Calculate  $A_{(532-600)}$  [71]. The MDA concentration was estimated using the Beer–Lambert–Bouguer law, MDA extinction coefficient  $\epsilon_{532} 155 \text{ mM}^{-1} \text{ cm}^{-1}$ ; the amount of MDA was calculated; and the values were normalized to the fresh weight of each sample.

#### 4.5. Determination of Total Phenolic Content

Total phenolic content (TPC) in carob leaf extracts was determined using the Folin–Ciocalteu colorimetric method [72,73]. Dry tissue (0.1 g) from 10 leaf samples from each treatment was ground in an electric mill (Janke & Kunkel-Mikro-Feinmuhle-Cullati, IKA Labortechnik, Wasserburg, Germany) and immersed in 10 mL MeOH (50% v/v). The tissue was incubated in a water bath for 3 h at 40 °C and vortexed regularly. Phenolic compounds were extracted, filtered using Whatman® (Maidstone, UK) #2 filter paper, and kept tightly sealed at 4 °C overnight. Furthermore, an aliquot (0.05 mL) of the diluted leaf extract [1:5 MeOH 10% (v/v)] was added to a solution consisting of 3.95 mL of dH<sub>2</sub>O, 0.25 mL Folin–Ciocalteu (Sigma-Aldrich™, Milan, Italy) reagent (that was previously diluted with water 1:10 v/v), 0.75 mL Na<sub>2</sub>CO<sub>3</sub> 20% (w/v) and was vortexed for 30 s. The solution was kept at 20 °C for 2 h and the absorption of the resulting colorimetric reaction was measured with a UV–VIS spectrophotometer (Pharmacia Biotech Novaspec II, Uppsala, Sweden) at 760 nm. Calculation of the total phenolic content was performed using standard curves of tannic acid and expressed as mg of tannic acid equivalent per g (dry weight) of leaf tissues.

To create the standard curve, the procedure was as follows; 0.05 mL of the different tannic acid concentrations, that is, 0.02 mg/mL, 0.08 mg/mL, 0.16 mg/mL, and 0.40 mg/mL, were added to 3.95 mL of dH<sub>2</sub>O, 0.25 mL Folin–Ciocalteu reagent (previously diluted with water 1:10 *v/v*), 0.75 mL Na<sub>2</sub>CO<sub>3</sub> 20% (*w/v*), and vortexed. The absorbance of these solutions, following the colorimetric reaction (after 2 h at 20 °C), was measured with a UV–VIS spectrophotometer (Pharmacia Biotech Novaspec II, Uppsala, Sweden) at 760 nm; the absorbance was plotted versus the concentration for the standard curve.

#### 4.6. Mass Spectroscopy (HRMS) Analysis

Leaves of *C. siliqua* L. were dried at room temperature and ground to a fine powder using a laboratory blender to achieve sample uniformity and protect the initial raw material from infections and chemical degradations. Then, extractions were carried out via ultrasound-assisted extraction (UAE). In brief, 3 g of each sample were mixed in a ratio of 1:10 with solvent and kept in the ultrasound bath for 20 min, at 30 °C. Three successive extractions were performed for each sample. Initially, samples were extracted with dichloromethane (DCM), then methanol (MeOH), and finally with water (H<sub>2</sub>O). Samples were filtered and evaporated until dryness. Extractions were performed in triplicate for each sample, while aliquots were used for the subsequent analyses. Furthermore, the DCM fraction was subjected to an additional liquid–liquid extraction step with ethyl acetate and n-hexane (EtOAc/n-Hex 1:1 *v/v*). The EtOAc extracts were concentrated until dry in a rotary evaporator under reduced pressure at 30 °C followed by their analysis with UPLC-ESI (±) Orbitrap-MS. Additionally, aliquots of the n-hex fraction were used for the subsequent GC-MS analyses.

UPLC-HRMS and HRMS/MS analysis was conducted on a Waters Acquity® UPLC system (Waters Corporation, Milford, MA, USA) coupled with an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, Waltham, MA, USA). To optimize UPLC conditions, eluants, column temperature, and column properties were examined. Improved peak shape was achieved with a combination of acetonitrile (ACN), acidified water, and a column temperature of 40 °C. The mass spectrometer, equipped with an electrospray ionization (ESI) source, operated in both negative and positive modes. The extracts were analyzed with an LC gradient consisting of H<sub>2</sub>O with 0.1% formic acid (FA) (solvent A) and ACN (solvent B). The samples were dissolved in the mobile phase (90% B and 10% A). The gradient elution was conducted with 95% (A) and 5% (B) as initial composition and was maintained for 1 min. For the next 15 min, the organic phase (B) was increased to 100% and this composition remained for 2 min before the initial conditions were restored for equilibration purposes. The total running time was 20 min. Throughout the run, the flow rate was maintained at 400 µL min<sup>−1</sup> and the injection volume was set to 10 µL.

High-resolution mass spectra were acquired in the full scan *m/z* range of 113–1000, in negative and positive mode at a resolving power of 30,000 and data-dependent MS/MS events were acquired, using an electrospray ionization source (ESI). In both modes, the data-dependent acquisition involved collision-induced dissociation C-trap (CID) with a normalized collision energy set at 35 V and a mass resolution of 10,000. In negative mode capillary temperature was adjusted to 350 °C and the source voltage was 2.7 kV, with tube lens and capillary voltage tuned at −100 V and −30 V, respectively. In positive mode capillary temperature was set to 350 °C and the source voltage was 3.60 kV, with tube lens and capillary voltage tuned at +120 V and +40 V, respectively. System control and spectral interpretation were carried out using the Xcalibur™ (Version 2.2, Thermo Scientific) software. For identification and dereplication, the following ranking criteria were applied: *Rt*, *EC*,  $10 \leq \text{RDBeq} \leq 20$ , mass range *m/z* 100–1000 Da, mass tolerance  $\leq$

5 ppm; HRMS/MS spectra of the selected peaks and identification of the fragmentation pattern, as well as literature references were consulted for further confirmation.

#### 4.7. Data Processing and Chemometrics

After LC-HRMS/MS acquisition, the data was processed in order to be subjected to multivariate data analysis (MVA) for the generation of descriptive statistical models. HRMS/MS data was recorded with Xcalibur 2.2. and raw files (.raw, Thermo) were imported to MZmine 2.53 software for data processing. The peak list was generated with a centroid selection algorithm. For chromatogram building of the generated mass list, 0.05 min was set as the minimum time of span and 5 ppm for mass tolerance. A chromatogram deconvolution module was employed, and spectra were processed with a local minimum search algorithm. The minimum retention time range was set at 0.1 min and the peak width at 0.05–0.7 min. Chromatograms were aligned, and spectra were normalized regarding retention time with 0.005 min tolerance. The joint aligner algorithm was applied to detected masses using a match score, calculated based on the mass and detection time of each peak, was used. Finally, gap filling was implemented, using the peak finder method.

Both peak lists (positive and negative mode) were imported to SIMCA 14.1 (Umetrics, Umeå, Sweden) software for statistical analysis. Mainly, Principal Component Analysis (PCA) and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) were implemented for sample visualization and discrimination, while s-plots were built between groups for determination and identification of statistically significant metabolites responsible for the observed trends and classifications. For this purpose,  $p$ -values in PCA and Variable Importance in Projection (VIP) values of OPLS-DA models, which rank variable contribution, were estimated, and evaluated.  $p < 0.05$  and VIP scores  $> 1$  were considered statistically significant. The generated models were evaluated for their  $R^2$  and  $Q^2$  parameters indicating the goodness of fit and predictability, respectively. Only models with  $R^2$  values close to 1,  $Q^2$  values over 0.5, or models with lower  $R^2$  but close to  $Q^2$  value were accepted. A permutation test was also applied for further validation of the models. Similarly, only models that succeeded in the permutation test were used for data visualization and subsequent VIP calculations.

#### 4.8. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Carob leaves n-Hex extracts underwent chemical analysis utilizing gas chromatography–mass spectrometry (GC-MS) using an Agilent 8890 GC system (Agilent Technologies, Santa Clara, CA, USA), equipped with an Agilent 7693A autosampler (Agilent, Santa Clara, CA, USA), an HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25  $\mu$ m film thickness) and an Agilent 5977B GC/MSD mass spectrometer. Helium served as the carrier gas with a flow rate of 1 mL/min. Column temperature initiated at 60 °C for 5 min, then gradually increased to 280 °C at a rate of 3 °C/min, maintaining that temperature for an additional 10 min. Electron ionization (EI) with an ionization energy of 70 eV was employed for MS detection. Prior to injection to the GC-MS, 0.4 mg of each extract was diluted in 1 mL of n-hexane and sent for analysis. The injection volume was 1.0  $\mu$ L and diluted samples were injected automatically in splitless mode. Freshly prepared samples in triplicate were analyzed just before the analysis. Alkane C8–C20 in hexane served as a standard marker. Data was acquired and processed using MassHunter Workstation Qualitative Analysis Software (version 10.0). Compound identification involved comparing obtained mass spectra with available spectral libraries (Adams07, Nist20, and Wiley275),

as well as through comparison of their relative retention indices (RRIs) with ones found in the literature.

#### 4.9. Strains of Microorganisms and Growth Conditions

Bacterial strains included *Bacillus subtilis* DSM10, *Escherichia coli* DSM6897, *Pseudomonas aeruginosa* DSM50071, *Candida albicans* DSM1386, *Staphylococcus aureus* DSM346, *Saccharomyces cerevisiae* DSM1333, and *Xanthomonas campestris* pv. *campestris* 1656 BPIC, *Pseudomonas syringae* pv. *syringae*, *Erwinia amylovora* 842 BPIC (*X. campestris* pv. *campestris* 1656 BPIC, *P. syringae* pv. *syringae*, *E. amylovora* 842 BPIC were obtained from the Benaki Phytopathological Institute Collection (BPIC) (<https://cc.info.wdcm.org>) (Table 4).

**Table 4.** Indicator strains, growth, and culture conditions.

| Indicator Strains                                   | Media                         | Incubation Temperature | Accession Number |
|-----------------------------------------------------|-------------------------------|------------------------|------------------|
| <i>Bacillus subtilis</i>                            | Luria-Bertani (LB) agar/broth | 30 °C                  | DSM10            |
| <i>Escherichia coli</i>                             | Luria-Bertani (LB) agar/broth | 37 °C                  | DSM6897          |
| <i>Pseudomonas aeruginosa</i>                       | Luria-Bertani (LB) agar/broth | 30 °C                  | DSM50071         |
| <i>Candida albicans</i>                             | YPD agar/broth                | 37 °C                  | DSM1386          |
| <i>Staphylococcus aureus</i>                        | Luria-Bertani (LB) agar/broth | 37 °C                  | DSM346           |
| <i>Saccharomyces cerevisiae</i>                     | YPD agar/broth                | 30 °C                  | DSM1333          |
| <i>Xanthomonas campestris</i> pv. <i>campestris</i> | Luria-Bertani (LB) agar/broth | 30 °C                  | 1656 BPIC        |
| <i>Pseudomonas syringae</i> pv. <i>syringae</i>     | Luria-Bertani (LB) agar/broth | 30 °C                  | -                |
| <i>Erwinia amylovora</i>                            | Luria-Bertani (LB) agar/broth | 30 °C                  | 842 BPIC         |

#### 4.10. Broth Microdilution Method

The broth microdilution technique was used to determine the antimicrobial potential of the extracts against the nine indicator strains, as given in Table 4. The method was followed as described [74] with the following alterations: DMSO 10% (*w/w*) was used as reference bactericide and the cytotoxicity of the solvents was also tested, at the maximum concentration they had in the final solutions. All plates were incubated for 24 h and OD absorbance (600 nm) was measured at 0 and 24 h after inoculation in triplicate with 10 s shaking UV/VIS on a monochromatic microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific Corporation: Waltham, MA, USA). The effect of the extracts on the growth of the indicator strains was evaluated by the reduction or not of the growth of the indicator strains, in the presence of 100 µg/mL, 200 µg/mL, and 500 µg/mL of the extracts.

#### 4.11. PCA Analysis

For the statistical analysis of chlorophyll-a and -b, MDA, and TPC. All data was expressed as the mean of 10 samples  $\pm$  standard error of the mean. From each treatment, 20 leaves (2 leaves per seedling) were used per replication; 20 leaves from pot tray 1 and 20 leaves from pot tray 2. All data were expressed as the mean of 2 replicates  $\pm$  standard error of the mean ( $N = 20$ ,  $n = 2$ ). Data were previously checked for their normality, while the Tukey test was evaluated using OriginPro v.9.1 and MS Excel, for the statistical significance. Then data was subjected to Principal Component Analysis (PCA) using Python and the scikit-learn library [75] to reduce data dimensionality and identify primary variance components.

## 5. Conclusions

Carob is one of the most common trees of the Mediterranean basin and has been a part of the culture and eating habits for thousands of years. Our data demonstrate that no “impressive” changes were observed in the morphology, anatomy, and physiology of cold- or heat-stressed samples. Furthermore, based on the metabolite profiling approach and MVA, it seems that there is an impact on metabolome under stress conditions, and certain marker compounds were revealed as belonging to tannins and flavonoids. Moreover, non-polar/volatile compounds belonging to  $\alpha$ -tocopheroids, fatty alcohols, sulfones, and triterpenoids were found to be altered either in high or low temperatures. However, the carob tree presents only a small shift of its metabolism to anticipate stressing conditions. Furthermore, the MeOH-H<sub>2</sub>O (MW-polar constituents) leaf extracts presented notable activity against the human pathogens *Candida albicans* and *Saccharomyces cerevisiae*, while DCM (D-non-polar constituents) extracts inhibited the growth of the phytopathogenic *Erwinia amylovora*. This data, along with recent [76], demonstrated that carob trees can grow in different polluted environments, indicating that *C. siliqua* is the most adapted Mediterranean plant, with “sophisticated” reactions. This aspect could upgrade the concept of the carob tree as a useful plant for green city creation along with its use in poor/drought soils and arid/semi-arid environments currently, as climate change is becoming a crisis.

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## Article

# *Artemisia herba-alba* Essential Oil: Chemical Composition, Phytotoxic Activity and Environmental Safety

Beáta Baranová <sup>1</sup>, Daniela Gruľová <sup>1</sup>, Flavio Polito <sup>2,\*</sup>, Vincent Sedlák <sup>3</sup>, Mária Konečná <sup>3</sup>, Marta Mydlárová Blaščáková <sup>3</sup>, Ismail Amri <sup>4</sup>, Vincenzo De Feo <sup>1</sup> and Janka Poráčová <sup>3</sup>

<sup>1</sup> Department of Ecology, Faculty of Humanities and Natural Sciences, University of Prešov, 17 Novembra 1, 08001 Prešov, Slovakia; beata.baranova@unipo.sk (B.B.); daniela.grulova@unipo.sk (D.G.); defeo@unisa.it (V.D.F.)

<sup>2</sup> Department of Pharmacy, University of Salerno, Via Giovanni Paolo II. 132, Fisciano, 84084 Salerno, Italy

<sup>3</sup> Department of Biology, Faculty of Humanities and Natural Sciences, University of Prešov, 17 Novembra 1, 08001 Prešov, Slovakia; vincent.sedlak@unipo.sk (V.S.); maria.konecna@unipo.sk (M.K.); marta.blascakova@unipo.sk (M.M.B.); janka.poracova@unipo.sk (J.P.)

<sup>4</sup> Laboratory of Biotechnology and Nuclear Technology, National Center for Nuclear Sciences and Technologies (CNSTN), Sidi Thabet 2020, Tunisia; amri\_amri@live.fr

\* Correspondence: fpolito@unisa.it

**Abstract:** Weeds cause a decrease in the quantity and quality of agricultural production and economic damage to producers. The prolonged use of synthetic pesticides causes problems of environmental pollution, the possible alteration of agricultural products and problems for human health. For this reason, the scientific community's search for products of natural origin, which are biodegradable, safe for human health and can act as valid alternatives to traditional herbicides, is growing. Essential oils can have useful implications in agriculture by acting as effective alternatives to chemical herbicides. In this work, the chemical composition of an EO from *Artemisia herba-alba* and its herbicidal properties were studied on two weeds (*Lolium multiflorum* and *Trifolium pratense*) and two crops (*Brassica napus* and *Hordeum vulgare*) and its environmental safety was also assessed using three model organisms: *Chaoborus* sp., *Tubifex tubifex* and *Eisenia foetida*. The principal component of the EO was camphor (26.02%), with  $\alpha$ - and  $\beta$ -thujone (9.60 and 8.38%, respectively), 1,8-cineole (8.02%), piperitenone (5.29%) and camphene (4.95%) as the main components. The EO demonstrated variable phytotoxic effects with a dose-dependent manner, inhibiting both the germination and the radical elongation of the tested seeds, and was also found to be environmentally safe for the selected organisms. The results lay the foundation for considering this EO as a potential weed control agent.

**Keywords:** *Artemisia herba-alba*; essential oil; composition; phytotoxicity; environmental safety

## 1. Introduction

A serious problem that afflicts the world of agricultural production and with which growers must continuously fight is that of weeds. The growth of weed plants within cultivated territories intended for production for human consumption causes a decrease in the quantity and quality of agricultural production, disturbing the growth of desired products and therefore causing economic damage to producers [1]. To address this problem, a series of chemical and synthetic herbicides have been developed over the years. However, although they act effectively, their massive and prolonged use causes environmental pollution, both of soil and water, and the possible alteration of agricultural products, with a consequent potential risk to human health [2].

For this reason, there is a growing interest among the scientific community in the search for products of natural origin, which are biodegradable, safe for both the environment and human health and can act as valid alternatives to traditional herbicides [3]. Plants produce secondary metabolites—bioactive compounds not directly involved in primary growth and development processes, but with significant ecological roles. Among them, some influence the growth of other plants, generating positive or negative effects known as allelopathy. This phenomenon, mediated by chemicals released into the environment, contributes to the regulation of plant interactions and ecosystem dynamics [4]. This phenomenon may have useful implications in the agricultural field as existing processes in nature can be exploited to search for valid alternatives to chemical herbicides, thus avoiding their demonstrated toxicity [3]. Essential oils (EOs) are complex mixtures of molecules, mainly volatile, capable of exerting numerous biological activities. They are used by plants as a means of communication and interaction and can be released into the environment to influence the behavior of other plants [5,6]. Although EOs can exert significant biological effects, they are generally not considered toxic at the concentrations used for therapeutic, cosmetic or environmental purposes, and they have low persistence in the environment and do not accumulate in food chains [7]. However, it should not be overlooked that their characteristics such as the hydrophobicity and prolonged integrity of the vesicles can have a significant impact on the environment on organisms present therein. For this reason, it is useful to evaluate the possible ecotoxic effects that the application of an EO in nature could have [8,9].

*Artemisia herba-alba* Asso, also known as desert wormwood, is a resilient, perennial dwarf shrub belonging to the Asteraceae family. It grows in arid and semi-arid areas of Spain, North Africa and the Middle East [10]. It plays an important role in the traditional medicine of various cultures due to its numerous therapeutic properties: antidiabetic, antihypertensive, antioxidant, antifungal, antimalarial, antispasmodic and many others [11–16].

The available literature reports significant variability in the composition of the EO of *A. herba-alba*, depending on the geographical region, the harvest period or the specific plant organ used for the EO extraction [17,18]. In general, the EOs from different locations in North Africa such as Algeria and Tunisia were found to belong to the chemotype thujone, camphor and, more rarely, eucalyptol [19,20]. Among the various components, thujone is the one that raises the most concerns due to its harmfulness. The World Health Organization (WHO) [21] and the Scientific Committee on Food of the European Commission [22] conducted a review on the acute and chronic toxicity of thujone. The European Parliament and Council regulation [23] prohibited the use of chemically pure thujone directly added to foods, though it may be indirectly introduced through the use of thujone-containing plants. Thus, thujone is banned as a food additive in the USA and its presence in foods and beverages is regulated in several countries [22]. The content and biological effects of thujone have been investigated using plant materials from *Artemisia absinthium* L., *Salvia officinalis* L., *Tanacetum vulgare* L. and *Thuja occidentalis* L. [19,22,24]. Since the 1800s, thujone has been recognized as a potent neurotoxin. Risk assessment for thujone, a primary component in herbal formulations, involves substantial uncertainties and challenges [25].  $\alpha$ -Thujone exhibits neurotoxic effects in rats and has antinociceptive activity in mice, and cases of human poisoning have been linked to the ingestion of wormwood oil containing  $\alpha$ -thujone [26–29]. This monoterpenoid occurs in many plants, including different *Artemisia* species. In *Artemisia absinthium* L. and herbal medicines,  $\alpha$ -thujone acts as a fast-acting and quickly detoxified modulator of the GABA-gated chloride channel [30,31]. Wormwood extracts have been used for controlling gastrointestinal worms, with documented use dating back to ancient Egypt. *A. absinthium* and wormwood oil exhibit insecticidal properties, with  $\alpha$ -thujone identified as one of the two most toxic monoterpenoids tested against larvae of the western corn rootworm [32,33]. In addition to neurotoxic effects,

thujones may exhibit genotoxic and carcinogenic properties; however, they can also demonstrate antimutagenic, immunomodulatory and anticarcinogenic effects [34–38]. Growing public distrust of synthetic drugs and pesticides has increased the popularity of naturally occurring drugs and herbicides, even though these alternatives are not subjected to the same rigorous safety testing and toxicological evaluation [39–41].

In this work, the chemical composition of an EO from Tunisian *A. herba-alba* and its herbicidal properties were studied on two globally important weeds (*Lolium multiflorum* Lam., annual ryegrass and *Trifolium pratense* L., red clover) and two crops (*Brassica napus* L., rapeseed and *Hordeum vulgare* L., barley). Due to the significant presence of thujone in the EO, three model organisms (*Chaoborus* spp., *Tubifex tubifex* and *Eisenia foetida*), important indicators of water and soil health and representatives of aquatic, semi-aquatic and soil organisms, were exposed to the identical concentrations of EO applied in the phytotoxicity test. In the discussion section (Section 3), it is seen how the results of this work are partially in agreement with what is present in the literature regarding the chemical composition of the EO; in fact, compounds such as camphor and thujone are recognized among the main components in many EOs of *A. herba-alba*. The results are in strong agreement regarding the phytotoxic activity, although the seeds considered are different, the EO of *A. herba-alba* being recognized to have strong phytotoxic activity. The results provided information on the phytotoxic potential of the EO and its possible ecotoxic impact.

## 2. Results

### 2.1. Chemical Composition

The EO analysis highlighted the presence of 81 compounds, accounting for 97.98% of the total. The main class was that of oxygenated monoterpenes (79.35%). The compound present in the highest quantity was camphor (26.02%), the other main components being  $\alpha$ - and  $\beta$ -thujone (9.60 and 8.38%, respectively), 1,8-cineole (8.02%), piperitenone (5.29%) and camphene (4.95%) (Table 1).

**Table 1.** Chemical composition of the EO.

|    |                         | %    | Retention Time (min) | Ki <sup>a</sup> | Ki <sup>b</sup> | Identification <sup>c</sup> |
|----|-------------------------|------|----------------------|-----------------|-----------------|-----------------------------|
| 1  | Santolina triene        | 0.18 | 11.116               | 836             | 1036            | 1, 2                        |
| 2  | Tricyclene              | 0.32 | 11.638               | 843             | 1012            | 1, 2                        |
| 3  | $\alpha$ -Pinene        | 0.99 | 12.472               | 854             | 1025            | 1, 2, 3                     |
| 4  | Camphene                | 4.95 | 13.456               | 867             | 1068            | 1, 2, 3                     |
| 5  | Sabinene                | 0.74 | 15.199               | 874             | 1122            | 1, 2                        |
| 6  | Dehydro-1,8-cineole     | 0.06 | 16.401               | 905             | 1192            | 1, 2                        |
| 7  | Mesitylene              | 0.28 | 16.709               | 909             | 1251            | 1, 2                        |
| 8  | $\alpha$ -Phellandrene  | 0.06 | 17.341               | 917             | 1168            | 1, 2, 3                     |
| 9  | Yomogi alcohol          | 0.20 | 17.616               | 920             | 1395            | 1, 2                        |
| 10 | $\alpha$ -Terpinene     | 0.13 | 18.241               | 929             | 1178            | 1, 2, 3                     |
| 11 | 1,2,4-Trimethyl benzene | 0.17 | 18.605               | 933             |                 | 1, 2                        |
| 12 | o-Cymene                | 0.87 | 18.954               | 938             | 1310            | 1, 2                        |
| 13 | 1,8-cineole             | 8.02 | 19.311               | 942             | 1211            | 1, 2, 3                     |
| 14 | Santolina alcohol       | 0.25 | 19.989               | 951             |                 | 1, 2                        |
| 15 | $\gamma$ -Terpinene     | 0.15 | 21.344               | 969             | 1245            | 1, 2, 3                     |
| 16 | Terpinolene             | 1.11 | 21.527               | 971             | 1282            | 1, 2, 3                     |
| 17 | trans-Sabinene hydrate  | 0.29 | 22.165               | 979             | 2092            | 1, 2                        |



Table 1. Cont.

|    |                                          | %     | Retention Time (min) | Ki <sup>a</sup> | Ki <sup>b</sup> | Identification <sup>c</sup> |
|----|------------------------------------------|-------|----------------------|-----------------|-----------------|-----------------------------|
| 18 | $\alpha$ -Thujone                        | 9.60  | 24.74                | 1010            | 1423            | 1, 2, 3                     |
| 19 | Santolina epoxide                        | 0.18  | 25.002               | 1013            |                 | 1, 2                        |
| 20 | $\beta$ -Thujone                         | 8.38  | 25.713               | 1023            | 1440            | 1, 2, 3                     |
| 21 | Chrysantenone                            | 0.81  | 26.078               | 1027            | 1508            | 1, 2                        |
| 22 | <i>trans</i> - <i>p</i> -Menth-2-en-1-ol | 0.72  | 26.404               | 1032            | 1584            | 1, 2                        |
| 23 | <i>cis</i> -Verbenol                     | 0.07  | 26.812               | 1037            | 1660            | 1, 2                        |
| 24 | Camphor                                  | 26.02 | 27.518               | 1047            | 1515            | 1, 2, 3                     |
| 25 | Camphene hydrate                         | 0.21  | 27.902               | 1052            | 1602            | 1, 2                        |
| 26 | $\beta$ -Pinene oxide                    | 0.11  | 28.454               | 1059            | 1364            | 1, 2                        |
| 27 | Pinocarvone                              | 1.46  | 28.752               | 1063            | 1576            | 1, 2                        |
| 28 | Borneol                                  | 3.60  | 29.14                | 1069            | 1670            | 1, 2, 3                     |
| 29 | <i>p</i> -Mentha-1,5-dien-8-ol           | 0.18  | 29.47                | 1073            | 1674            | 1, 2                        |
| 30 | Terpinen-4-ol                            | 0.53  | 29.861               | 1078            | 1601            | 1, 2, 3                     |
| 31 | $\alpha$ -Thujenal                       | 0.09  | 30.278               | 1084            |                 | 1, 2                        |
| 32 | ( <i>E</i> )-Isocitral                   | 0.32  | 30.396               | 1085            |                 | 1, 2                        |
| 33 | Myrtenal                                 | 1.00  | 30.846               | 1091            | 1632            | 1, 2                        |
| 34 | <i>cis</i> -Piperitol                    | 0.13  | 31.13                | 1095            | 1750            | 1, 2                        |
| 35 | Myrtenol                                 | 0.25  | 31.265               | 1097            | 1790            | 1, 2                        |
| 36 | Verbenone                                | 0.76  | 31.942               | 1100            | 1726            | 1, 2                        |
| 37 | <i>trans</i> -Piperitol                  | 0.27  | 32.079               | 1102            | 1710            | 1, 2                        |
| 38 | <i>trans</i> -Carveol                    | 0.10  | 32.303               | 1105            | 1720            | 1, 2                        |
| 39 | <i>cis</i> -Carveol                      | 0.06  | 32.98                | 1115            | 1854            | 1, 2                        |
| 40 | ( <i>E</i> )-Ocimenone                   | 0.17  | 33.159               | 1117            |                 | 1, 2                        |
| 41 | Car-3-en-2-one                           | 1.49  | 34.405               | 1135            |                 | 1, 2                        |
| 42 | Piperitone                               | 0.48  | 35.181               | 1146            | 1730            | 1, 2                        |
| 43 | <i>cis</i> -Verbenyl acetate             | 0.83  | 35.692               | 1153            |                 | 1, 2                        |
| 44 | <i>p</i> -Mentha-1,8-dien-3-one          | 1.62  | 36.41                | 1164            |                 | 1, 2                        |
| 45 | Bornyl acetate                           | 0.79  | 37.241               | 1176            | 1579            | 1, 2                        |
| 46 | <i>trans</i> -Pinocarvyl acetate         | 0.10  | 37.865               | 1184            | 1661            | 1, 2                        |
| 47 | <i>cis</i> -Pinocarvil acetate           | 0.28  | 38.262               | 1190            |                 | 1, 2                        |
| 48 | ( <i>Z</i> )-Patchenol                   | 0.62  | 38.573               | 1195            |                 | 1, 2                        |
| 49 | ( <i>E</i> )-Patchenol                   | 2.80  | 39.504               | 1203            |                 | 1, 2                        |
| 50 | $\delta$ -Elemene                        | 0.32  | 40.493               | 1218            | 1469            | 1, 2                        |
| 51 | <i>cis</i> -Carvyl acetate               | 0.70  | 41.82                | 1238            |                 | 1, 2                        |
| 52 | $\alpha$ -Copaene                        | 0.18  | 42.891               | 1254            | 1491            | 1, 2                        |
| 53 | Piperitenone                             | 5.29  | 44.78                | 1282            | 1909            | 1, 2                        |
| 54 | Daucene                                  | 0.59  | 45.562               | 1294            | 1495            | 1, 2                        |
| 55 | $\beta$ -Cubebene                        | 0.25  | 46.187               | 1297            | 1542            | 1, 2                        |
| 56 | $\beta$ -Longipinene                     | 0.10  | 47.113               | 1312            |                 | 1, 2                        |
| 57 | $\beta$ -Cedrene                         | 0.12  | 47.624               | 1321            |                 | 1, 2                        |
| 58 | Aromadendrene                            | 0.12  | 48.052               | 1328            | 1620            | 1, 2                        |
| 59 | <i>allo</i> -Aromadendrene               | 0.24  | 49.139               | 1345            | 1660            | 1, 2                        |



Table 1. Cont.

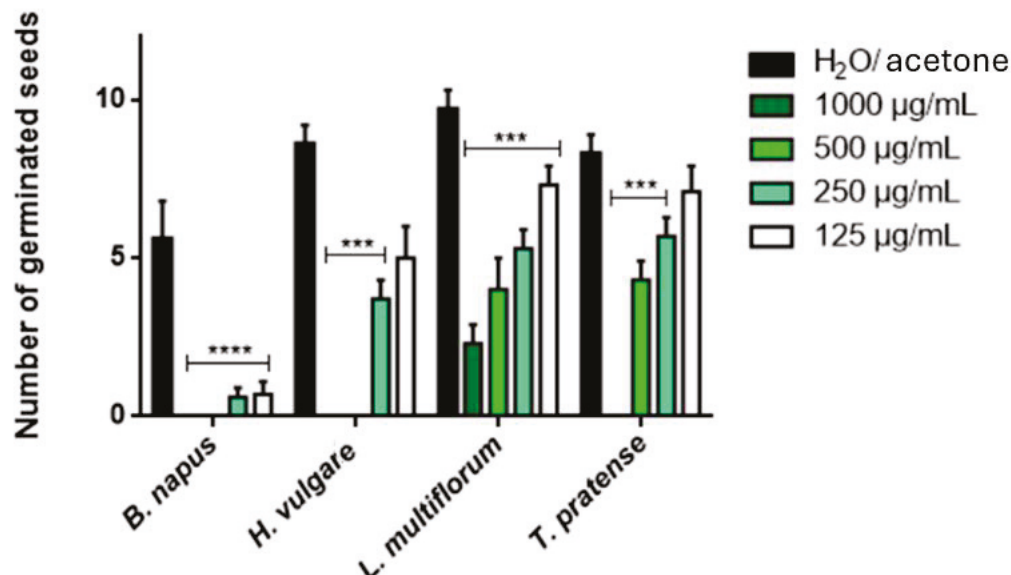
|                             |                                                                       | %     | Retention Time (min) | Ki <sup>a</sup> | Ki <sup>b</sup> | Identification <sup>c</sup> |
|-----------------------------|-----------------------------------------------------------------------|-------|----------------------|-----------------|-----------------|-----------------------------|
| 60                          | Germacrene D                                                          | 0.38  | 49.337               | 1348            | 1708            | 1, 2                        |
| 61                          | Chrysantenyl 2-methylbutanoate                                        | 0.38  | 49.871               | 1357            |                 | 1, 2                        |
| 62                          | $\delta$ -Guaiene                                                     | 0.35  | 50.267               | 1363            |                 | 1, 2                        |
| 63                          | Bornyl 2-methylbutanoate                                              | 0.07  | 50.986               | 1375            | 1241            | 1, 2                        |
| 64                          | $\delta$ -Amorphene                                                   | 0.19  | 52.007               | 1391            |                 | 1, 2                        |
| 65                          | 5-Isopropenyl-2-methyl-2-cyclohexen-1-yl pivalate                     | 0.06  | 52.784               | 1399            |                 | 1, 2                        |
| 66                          | Palustrol                                                             | 0.28  | 54.352               | 1426            | 1930            | 1, 2                        |
| 67                          | Spathulenol                                                           | 1.24  | 55.062               | 1438            | 2127            | 1, 2                        |
| 68                          | Caryophyllene oxide                                                   | 0.70  | 55.211               | 1446            | 1986            | 1, 2                        |
| 69                          | Viridiflorol                                                          | 0.82  | 55.801               | 1450            | 2090            | 1, 2                        |
| 70                          | <i>cis</i> -Carvyl angelate                                           | 0.21  | 56.04                | 1454            |                 | 1, 2                        |
| 71                          | <i>cis</i> -Eudesm-6-en-11-ol                                         | 0.24  | 56.403               | 1460            |                 | 1, 2                        |
| 72                          | Junenol                                                               | 0.07  | 57.18                | 1474            |                 | 1, 2                        |
| 73                          | $\gamma$ -Eudesmol                                                    | 0.33  | 57.896               | 1486            | 2176            | 1, 2                        |
| 74                          | Ylangenal                                                             | 0.23  | 58.196               | 1491            |                 | 1, 2                        |
| 75                          | Caryophylla-4(12),8(13)-dien-5 $\alpha$ -ol                           | 0.12  | 58.319               | 1493            | 2324            | 1, 2                        |
| 76                          | Isospathulenol                                                        | 0.15  | 58.505               | 1496            | 2228            | 1, 2                        |
| 77                          | $\tau$ -Cadinol                                                       | 0.22  | 58.746               | 1493            | 2151            | 1, 2                        |
| 78                          | $\alpha$ -Eudesmol                                                    | 0.34  | 59.026               | 1498            |                 | 1, 2                        |
| 79                          | $\alpha$ -Cadinol                                                     | 0.61  | 59.408               | 1505            | 2224            | 1, 2                        |
| 80                          | (1 <i>R</i> ,7 <i>S</i> )-Germacra-4(15),5,10(14)-trien-1 $\beta$ -ol | 0.18  | 61.115               | 1536            |                 | 1, 2                        |
| 81                          | Icosa-9,11-diyne                                                      | 0.10  | 72.287               | 1735            |                 | 1, 2                        |
| Total                       |                                                                       | 97.98 |                      |                 |                 |                             |
| Monoterpene hydrocarbons    |                                                                       | 9.95  |                      |                 |                 |                             |
| Oxygenated monoterpenes     |                                                                       | 79.35 |                      |                 |                 |                             |
| Sesquiterpene hydrocarbons  |                                                                       | 2.84  |                      |                 |                 |                             |
| Oxygenated sesquiterpenoids |                                                                       | 5.74  |                      |                 |                 |                             |
| Others                      |                                                                       | 0.10  |                      |                 |                 |                             |

<sup>a,b</sup> Kovats retention indices determined relative to a series of n-alkanes (C10–C35) on the apolar HP-5 MS and the polar HP Innowax capillary columns, respectively. <sup>c</sup> Identification method: 1 = comparison of the Kovats retention indices with published data, 2 = comparison of mass spectra with those listed in the NIST 02 and Wiley 275 libraries and with published data; 3 = co-injection with authentic compounds.

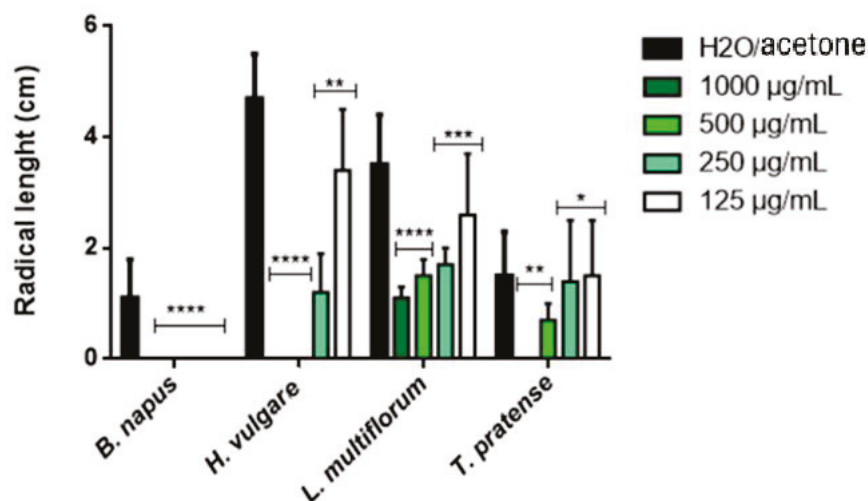
## 2.2. Phytotoxic Activity

Figures 1 and 2 report the herbicidal properties of the EO on the germination and radical elongation of the weeds, *Lolium multiflorum* and *Trifolium pratense*, and on the crops *Brassica napus* and *Hordeum vulgare*. The EO demonstrated variable phytotoxic effects in a dose-dependent manner. The EO was active on all the seeds tested both on the germination and on the radical elongation. However, the effect was slightly higher for the second event. At the highest concentrations (1000 and 500  $\mu\text{g/mL}$ ), the EO caused total inhibition (100%) of the germination of *B. napus* and *H. vulgare*. A 100% inhibition also occurs for *T. pratense* at the concentration of 1000  $\mu\text{g/mL}$  but not for the second higher concentration (48.20% of inhibition). For *L. multiflorum*, there are high inhibitions but not at 100%: 76.29% at

1000 µg/mL and 58.77% at 500 µg/mL. At the lowest concentrations (250 and 125 µg/mL), the effect was reduced: the highest inhibition values were found for *B. napus* (89.29 and 87.50%, respectively).



**Figure 1.** Phytotoxic activity of *A. herba-alba* EO against the germination of *B. napus*, *H. vulgare*, *L. multiflorum* and *T. pratense*. The results are the mean of three experiments  $\pm$  SD. \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.00001$  compared with the control (ANOVA followed by Dunnet's multiple comparison test).



**Figure 2.** Phytotoxic activity of *A. herba-alba* EO against the radical elongation of *B. napus*, *H. vulgaris*, *L. multiflorum* and *T. pratense*. The results are the mean of three experiments  $\pm$  SD. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.00001$  compared with the control (ANOVA followed by Dunnet's multiple comparison test).

The EO causes 100% inhibition of the radical elongation of *B. napus* at all concentrations, while at the highest concentrations (1000 and 500 µg/mL), it showed a total inhibition for *H. vulgaris* and a total inhibition for *T. pratense* only for the highest concentration. At the lowest concentrations, the activity significantly decreases. The highest inhibitions at 250 and 125 µg/mL were found for *B. napus* (100% for both concentrations), followed by *H. vulgaris* (74.47 and 27.66%, respectively) and *L. multiflorum* (51.42 and 25.71%, respectively). Instead, on *T. pratense*, the inhibition was 6.67% for the concentration 250 µg/mL and 0% at 125 µg/mL.

### 2.3. Ecotoxicity

The mortality of the model organisms after exposure to EO from *A. herba-alba* was assessed using the same concentrations as those used in the phytotoxicity test (1000 µg/mL, 500 µg/mL, 250 µg/mL and 125 µg/mL). The mortality index for the semi-aquatic organism *Tubifex tubifex* was 0% at all applied EO concentrations, even after prolonged exposure (assessments performed from 3 min up to 24 h). Furthermore, for the soil representative *Eisenia foetida*, no mortality was observed at any of the used concentrations after 48 h of exposure. Aquatic larvae of non-biting mosquitoes of *Chaoborus* spp. showed, after 48 h, an average mortality of 29% after exposure to the highest EO concentration used in the phytotoxicity tests. The second highest concentration caused a mortality of 8%. The lowest concentrations did not cause mortality for these aquatic organisms. All results were compared with a control test performed using distilled water, in which the survival of the organisms was 100%.

## 3. Discussion

Several papers are available concerning the chemical composition of EO from *A. herba-alba*, whose results are in partial agreement with what is highlighted in this work. Younsi and collaborators [42] studied the composition of the EOs from 80 individuals from 8 populations growing in different geographical areas of Tunisia. Four chemotypes (*trans*-sabinyl acetate,  $\alpha$ -thujone/*trans*-sabinyl acetate, camphor and  $\alpha$ -thujone/camphor/ $\beta$ -thujone) were identified, and oxygenated monoterpenes were the predominant class (73.6–89.7%). The composition is similar to that reported in this work, in which the EO belongs to the camphor chemotype, with the predominance of oxygenated monoterpenes and the presence of many of the main components highlighted in the work of Younsi, although in different amounts. An EO obtained by the SPME technique [43] was found to be rich in oxygenated monoterpenes (64.9%, slightly lower than the 79.35% present in this work), and among the main compounds, it presented  $\alpha$ -thujone (37.9%), germacrene D (16.5%), 1,8-cineole (8.4%),  $\beta$ -thujone (7.8%) and bicyclogermacrene (5.8%). Some of these are also present in the EO studied in this work:  $\alpha$ -thujone but in very different quantities (37.9 vs. 9.60%) and  $\beta$ -thujone and eucalyptol in similar quantities (7.8 vs. 8.38 and 8.4 vs. 8.02%, respectively). The same authors analyzed EOs from leaves, stems and roots of a Tunisian *A. herba-alba* [44]. All EOs present a considerable presence of  $\alpha$ -thujone (18.2–45.5%),  $\beta$ -thujone (5.7–11.4%), camphor (6.8–22.9%) and 1,8-cineole (1.7–8.3%). These compounds agree with the main compounds of the EO of this work, in very similar amounts (except  $\alpha$ -thujone, in lower amounts). In 2015, a comparative study was carried out on the composition of the EOs of *A. herba-alba* from different locations [45]. In all the EOs, oxygenated monoterpenes were the main class (65.5–70.3%), as in this work. The main components were camphor (0.64–31.51%), fenchol (7.51–13.85%), germacrene D (2.24–5.89%), limonene (4.57–7.29%), nordavanone (1.26–9.44%),  $\alpha$ -thujone (11.62–13.93%) and  $\beta$ -thujone (0.16–8.04%). Camphor,  $\alpha$ -thujone and  $\beta$ -thujone are also the main components in the EO studied in this work, with similar quantities except for  $\alpha$ -thujone (present in smaller quantities). Finally, there is a contribution regarding the EO composition of *A. herba-alba* from Spain [46], which reports as the main components camphor (15.0%) and 1,8-cineole (13.3%).

Few contributions are available regarding the possible herbicidal activity of the EO of *A. herba-alba*. The allelopathic potential of an EO from an Algerian *A. herba-alba* on nine weeds and two wheat varieties was tested. The results showed activity on all the seeds considered, but it varied both according to the seed and the concentrations used. The highest inhibitory activity, both on germination and radical elongation, was observed on *Silybum marianum* (L.) Gaertn., *Scorzonera laciniata* Jacq., *Mantisalca salmantica* (L.) Briw. &

Cavill., *Bromus madritensis* L., *Ammi visnaga* (L.) Lam. and *Diploaxis tenuifolia* (L.) DC., while it was lower on *Hordeum murinum* L. and *Sinapis arvensis* L. However, it had a stimulating effect on *Avena fatua* L. [6]. A strong inhibitory activity on the growth of crops such as *Lactuca sativa* L. and *Raphanus sativus* L. was instead highlighted for an EO from the leaves of *A. herba-alba*, able to completely inhibit the germination of these two species [43]. Finally, the phytotoxic properties of an EO of *A. herba-alba* on *Raphanus sativus*, *Lepidium sativum* L., *Sinapis arvensis*, *Triticum durum* L. and *Phalaris canariensis* L. seeds were reported [47]. The concentrations used were lower than those used in this work and the EO was inactive in inhibiting germination but more active in inhibiting radical elongation. At the highest doses, a stimulating effect on *R. sativus* was noted. These results agree with the greater activity of the EO regarding radical elongation rather than germination and show how effectively the EO has effects that manifest themselves on many weeds but also on some crops. EOs are recognized as effective phytotoxic agents [48] and act in this sense through mechanisms involving different communication and interaction signals between plants that, through phenotypic modifications, see their germination and root elongation processes influenced [49]. The activity is obviously linked to the composition: there are many volatile compounds active in this sense, and among these, some of the main components of the EOs studied in this article can contribute to phytotoxicity. In fact, camphor [28],  $\alpha$ - and  $\beta$ -thujone and 1,8-cineole have been reported for their phytotoxic activity [48–50]. There are no studies in the literature analyzing the ecotoxic potential of the EO of *A. herba-alba* using the organisms of this work. Although it is not excluded that *A. herba-alba* EO can pose some toxicity to non-target organisms, concentrations which impacted the germination or root growth of model plants within the phytotoxic bioassay were not toxic to soil nor to semiaquatic representatives at all. There was a slight mortality for aquatic organisms, which highlights the need for further studies and, in general, for caution in the practical application of EOs and adherence to correct application practices, just as with conventional herbicides [7–9]. The ecotoxicity effects manifested, although minimal, are linked to the composition and, in particular, to camphor [51–53]. Specific information on the ecotoxicity of thujone is not present in the literature. However, the works on this subject used different techniques and organisms, and therefore, a direct comparison with the present work is not yet possible.

## 4. Materials and Methods

### 4.1. Plant Materials

*Artemisia herba-alba* was collected at the beginning of September 2023 (flowering stage) in the city of Siliana (Bargou region, latitude 36°4'55" N longitude 9°22'29" E), North West of Tunisia. The plant was identified by Dr. I. Amri, and a voucher specimen is stored in the herbarium section of the Institut National de la Recherche en Génie Rural, Eau et Forest, INRGREF, Tunis. The plant was air-dried for 15 days and stored in a dark, humidity-controlled environment until it was ready for use.

### 4.2. Extraction of the EO

Aerial parts were steam-distilled for 2 h, according to the method reported by the European Pharmacopoeia [54]. The EO was solubilized in *n*-hexane, filtered over anhydrous sodium sulphate and stored under N<sub>2</sub> at +4 °C in the dark until tested and analyzed. The EO yield was 0.1% on dry weight.

#### 4.3. GC/MS Analyses

GC-MS analysis was performed using an Agilent 6850 Ser. II Apparatus (Santa Clara, CA, USA) equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm; 0.25 µm film thickness) and connected to an Agilent Mass Selective Detector (MSD 5973) (Santa Clara, CA, USA) with an ionization voltage of 70 V and an ion multiplier energy of 2000 V. The mass spectra were scanned in the range of 40–500 amu, with five scans per second. The chromatographic conditions were as reported above, and the transfer line temperature was 295 °C. The analysis was conducted on a scheduled basis: 5 min isothermally at 40 °C; subsequently, the temperature was increased by 2 °C/min until 270 °C, and finally, it was kept in isotherm for 20 min. The analysis was also performed on an HP Innowax column (50 m × 0.20 mm; 0.25 µm film thickness). In both cases, He was used as a carrier gas (1.0 mL/min). Most of the components were identified by comparing their Kovats indices (Ki) with those of the literature [55–58] and by a careful analysis of the mass spectra compared to those of the pure compounds available in our laboratory or to those present in the NIST 02 and Wiley 257 mass libraries [59]. The Kovats indices were determined in relation to a homologous series of n-alkanes (C10–C35), under the same operating conditions. For some compounds, the identification was confirmed by co-injection with standard samples. Components' relative concentrations were calculated by peak area normalization. Response factors were not considered.

#### 4.4. Phytotoxic Activity

The phytotoxic activity was evaluated against the seed germination and radicle emergence/elongation of two weeds, *Lolium multiflorum* Lam. and *Trifolium pratense* L., and two horticultural crops, *Brassica napus* L. and *Hordeum vulgare* L. The seeds were purchased from Blumen group srl (Emilia-Romagna, Italy). The seeds were surface-sterilized in 96% ethanol for 15 s and sown in Petri dishes (Ø = 90 mm) on three layers of Whatman filter paper. They were impregnated with 7 mL of deionized water, and then 7 mL of a water–acetone mixture (99.5:0.5, v/v) was used as the control since EOs were dissolved in this mixture due to their lipophilicity. Finally, 7 mL of the EO solution at different concentrations (1000, 500, 250 and 125 µg/mL) was tested. The controls carried out with the water–acetone mixture alone showed no differences in comparison to the controls in water alone. The germination conditions were 20 ± 1 °C, with a natural photoperiod. Seed germination was checked in Petri dishes every 24 h. A seed was considered germinated when the protrusion of the root became evident [60]. On the fifth day (after 120 h) for *B. napus* and *H. vulgare* and on the seventh day (after 168 h) for the other seeds, the effects on radicle elongation were determined by measuring the root length in cm. Each evaluation was replicated three times, using Petri dishes containing 10 seeds each. The data were expressed as the mean ± standard deviation for both germination and radicle elongation.

#### 4.5. Ecotoxicity

To test the ecotoxicity of EO, three model organisms, representing aquatic, semi-aquatic and soil organisms, were exposed to the same EO concentrations as applied in the phytotoxicity bioassay. Water was used as a control (0% mortality). Aquatic glassworms—larvae of the non-biting mosquito of *Chaoborus* spp. (Diptera: Chaoboridae)—are harmless representatives of the water invertebrates. They were subjected to the laboratory testing of mosquito larvicides following the standard methodology suggested by the World Health Organization [61], with a slight modification [62]. Mortality was evaluated after 24 h. For each EO concentration, 10 repetitions with 10 individuals were performed (n = 100).



Semiaquatic sludge worm *Tubifex tubifex* Müller, 1774 (Annelida, Oligochaeta: Tubificidae)—a standard model organism that is widely used in ecotoxicological studies—was tested for acute toxicity using an express 3 min test following Tichý et al. [63]. For each EO concentration, 10 repetitions with 10 individuals were performed ( $n = 100$ ).

As the zero mortality was observed after 3 min, prolonged exposition was applied and the number of death worms was checked after 10, 20, 30, 60, 180 and 240 min and then after 24 h.

The earthworm *Eisenia foetida* Savigny, 1826 (Annelida, Oligochaeta: Lumbricidae) was used as a representative of soil organisms. Ecotoxicity was tested using the contact method according to OECD [64]: the individuals were placed in a Petri dish on filter paper soaked with 2 mL of EO dilution, and mortality was evaluated after 48 h. For each EO concentration, 10 repetitions were performed ( $n = 10$  individuals).

#### 4.6. Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad Prism 6.0 (Software Inc., San Diego, CA, USA), expressed as the mean  $\pm$  standard deviation (S.D.). The results were compared to the untreated control and considered statistically significant, by Dunnett's test, when  $p < 0.05$  (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ).

### 5. Conclusions

In this study, data were collected regarding the composition, phytotoxicity and impact on environmental safety of the EO of *A. herba-alba* from Tunisia. The results demonstrate strong phytotoxicity activity both for what concerns the inhibitory action on germination and root elongation, especially for *T. pratense*. Furthermore, preliminary tests on a possible environmental toxicity gave encouraging results, since the EO did not demonstrate any danger for the reference organisms used. The results lay the foundation for considering the EO of *A. herba-alba* as a potential weed growth control agent that, at the same time, appears to have a less dangerous environmental impact than other chemical control agents.

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## Article

# Phytochemical Characterisation of *Sorbus* Species: Unveiling Flavonoid Profiles Related to Ploidy and Hybrid Origin

Emina Korić <sup>1,\*</sup>, Violeta Milutinović <sup>2</sup>, Alma Hajrudinović-Bogunić <sup>3</sup>, Faruk Bogunić <sup>3</sup>,  
Tatjana Kundaković-Vasović <sup>2</sup>, Irma Gušić <sup>1</sup>, Jelena Radović Selgrad <sup>2</sup>, Kemal Durić <sup>1</sup> and Haris Nikšić <sup>1</sup>

<sup>1</sup> Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina; irma.gusic@ffsa.unsa.ba (I.G.); kemal.duric@ffsa.unsa.ba (K.D.); haris.niksic@ffsa.unsa.ba (H.N.)

<sup>2</sup> Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11000 Belgrade, Serbia; violeta.milutinovic@pharmacy.bg.ac.rs (V.M.); tatjana.kundakovic@pharmacy.bg.ac.rs (T.K.-V.); jelena.radovic@pharmacy.bg.ac.rs (J.R.S.)

<sup>3</sup> Faculty of Forestry, University of Sarajevo, Zagrebačka 20, 71000 Sarajevo, Bosnia and Herzegovina; a.hajrudinovic@sfsa.unsa.ba (A.H.-B.); f.bogunic@sfsa.unsa.ba (F.B.)

\* Correspondence: emina.koric@ffsa.unsa.ba; Tel.: +387-33586170

**Abstract:** The genetic, morphological and taxonomic diversity of the genus *Sorbus* is due to homoploid and polyploid hybridisation, autopolyploidy and apomixis, which also influence the production and diversity of secondary metabolites, especially flavonoids. The aim of this study was to investigate the relationships and variations of flavonoids in terms of hybrid origin and ploidy level between the parental species and their hybrid derivatives. The sampling design included leaf material of the following *Sorbus* accessions from ten natural localities: parental taxa (di-, tri- and tetraploids of *S. aria*; diploid *S. torminalis* and *S. aucuparia*) and their di-, tri- and tetraploid hybrid derivatives from crosses of *S. aria* × *S. torminalis* (subg. *Tormaria*) as well as the tetraploid *S. austriaca* and *S. bosniaca*, which originate from crosses of *S. aria* × *S. aucuparia* (subg. *Soraria*). We analysed the flavonoid profiles from the leaf fractions by LC-MS. A total of 23 flavonoids were identified, including apigenin and luteolin derivatives, which distinguish the hybrid groups from each other. This profiling highlights the distinctiveness of the *Tormaria* and *Soraria* accessions and emphasises the potential of the subg. *Tormaria* for further research on bioactive compounds in biological studies.

**Keywords:** *Sorbus*; flavonoids; hybridisation; liquid chromatography; mass spectrometry; apigenin derivatives; luteolin derivatives; polyploidy

## 1. Introduction

Natural plant extracts contain different chemical profiles and corresponding biological activities with great application potential. In this regard, there is a growing interest in the discovery and production of plant extracts used in the pharmaceutical, cosmetic and food industries due to their bioactive compounds [1]. Since the biosynthetic pathway secondary metabolite formation varies within the same species due to different factors, a major challenge is to define all possible variability factors that may influence the composition of secondary metabolites [2]. The genus *Sorbus* L. is recognised as a highly valued source of bioactive compounds for the development of pharmaceutical, cosmetic and nutraceutical products [3] but has not yet been sufficiently explored.

The genus *Sorbus* (whitebeams, rowans and service trees) comprises tree and shrub species characterised by enormous genetic and morphological diversity. This diversity is

the result of polyploidisation, hybridisation and apomixis [4,5]. Recurrent hybridisation between diploid species of *S. aria* (L.) Crantz, *S. aucuparia* L., *S. chamaemespilus* (L.) Crantz and *S. torminalis* (L.) Crantz and backcrossing of hybrid derivatives with their parental species resulted in about 190–200 recognised species inhabiting Europe [6,7]. The genus is traditionally divided into five subgenera: (subg.) *Aria*, *Chamaemespilus*, *Cormus*, *Sorbus* and *Torminaria* [8]. However, phylogenetic analyses recognise at least two monophyletic groups (*Sorbus*, *Cormus*; *Aria*, *Torminaria* and *Chamaemespilus*) [9,10]. Due to the obvious polyphyletic nature of the genus and extensive hybridisation between the subgenera members, the *Sorbus* genus is divided into independent genera [7], but the traditional name is still in use.

Most species are polyploid, triploid and tetraploid and reproduce by facultative apomixis [4,5,8,11]. Most polyploid species are endemics with a narrow geographic distribution, whereas the parental species are much more widely distributed throughout Europe [12]. While many aspects of *Sorbus* taxonomy [6,8,11], genetics [4,13] and reproduction [5,14], including phytochemical diversity, have been thoroughly studied in different parts of Europe, they are still understudied in the Balkans.

The remarkable genetic, morphological and physiological variability within the genus *Sorbus* is also reflected in the production and diversity of secondary metabolites [15]. Polyploidy and hybridisation are considered triggers that cause increased productivity of secondary metabolites and their diversity [16]. In particular, hybridisation is a driving mechanism that facilitates the production of secondary metabolites and restores their combinations [17,18].

Recent studies have revealed a great diversity and variation in the phytochemical composition of the different *Sorbus* species [19–21]. The genus is rich in flavonoids including anthocyanins [21], phenolic acids [22,23], triterpenes/tannins [24] and various other phytochemicals known for their antioxidant properties [25,26], anti-inflammatory [27], antimicrobial [28], antihyperlipidemic [29], antidiabetic [30], neuroprotective [31], hepatoprotective [24] and cardioprotective effects [32].

Flavonoids, as a group of secondary metabolites, are useful indicators of hybridisation and, in some cases, polyploidisation [33,34], but recent studies do not support flavonoids as indicators of polyploidy. In previous phytochemical studies, well-known flavonols such as quercetin and kaempferol and their glycosides, along with their methyl derivatives and less common C-flavonoids, have been detected in various parts or organs of *Sorbus* taxa, including the species examined in this study: *S. aria*, *S. torminalis*, *S. aucuparia*, and *S. austriaca* [19,23,35–38]. Many studies in the literature have focused on the flavonoid content in *Sorbus* fruits or inflorescences [23,36–39], with fewer studies on the bark and even more limited research on the leaves [22,25]. It is interesting that leaf material is the least used in flavonoid research, even though leaves contain a much higher content of polyphenolic components compared to flowers and fruits [22,25]. Most studies investigated the phytochemical composition of crude methanolic extracts from fruits, inflorescences and leaves, while only two reported detailed phytochemical analysis of their fractions [21,39]. In most studies, the plant material used for the investigation of polyphenolic profiles also originated from cultivated *Sorbus* accessions from botanical gardens and parks [39,40]. Numerous studies dealing with the qualitative and quantitative analysis of *Sorbus* the polyphenolic compounds were based on LC-UV/VIS spectroscopy [39] and the Folin-Ciocalteu method [41]. In contrast, LC-MS [21,22], UPLC-QTOF-MS [19,42] and UPLC-ESI-MS [3] have been used in a few studies to determine the phytochemical profile of *Sorbus* species.



The aim of this study is to provide the first insights into the leaf flavonoid profiles of *Sorbus* species from the Balkans (Bosnia and Herzegovina) using liquid chromatography-mass spectrometry (LC-MS). We analysed the diploid *S. aria* (subg. *Aria* Pers.), *S. aucuparia* (subg. *Sorbus*) and *S. torminalis* (subg. *Torminaria*) as well as the members of the hybridogenous subg. *Soraria* Májovský and Bernátova (*S. aucuparia* × *S. aria*) and *Tormaria* Májovský and Bernátova (*S. torminalis* × *S. aria*). We included tetraploid *S. austriaca* (Beck.) Hedl. and *S. bosniaca* Hajrudinović, Frajman, Schönswetter and Bogunić from the subg. *Soraria*, as well as di-, tri- and tetraploids from the subg. *Tormaria*. In addition, we also included tri- and tetraploid samples of *S. aria*. In particular, our aim was to (1) identify flavonoid markers that distinguish *Sorbus* accessions; (2) investigate the influence of hybridisation on the variation in the qualitative composition of flavonoids; and (3) determine the relationships between the ploidy level and the qualitative and quantitative content of flavonoid compounds.

## 2. Results

### 2.1. Ploidy Level

The ploidy level of 17 individuals from 10 locations was determined (See Material and Methods). The absolute genome size revealed three ploidy levels. Diploid values ( $2C = 2x$ ) were found in *S. aucuparia* ( $2C = 1.43$  pg), *S. aria* ( $2C = 1.44$  pg), *S. torminalis* ( $2C = 1.44$  pg) and *S. torminalis* × *S. aria* ( $2C = 1.43$  pg). Triploids ( $2C = 3x$ ) of *S. aria* had  $2C = 2.11$  pg and *S. torminalis* × *S. aria* had  $2C = 2.18$  pg. Tetraploid cytotypes ( $2C = 4x$ ) were observed in *S. aria* ( $2C = 2.80$  pg), *S. austriaca* ( $2C = 2.79$  pg), *S. bosniaca* ( $2C = 2.80$  pg) and *S. torminalis* × *S. aria* ( $2C = 2.74$  pg). (See Material and Methods).

### 2.2. Mass Spectrometric Identification of Flavonoid Compounds

The structures of 23 flavonoids (1–23) were investigated using LC-MS by analysing 17 different ethyl acetate (EtOAc) fractions from methanol (MeOH) leaf extracts of various *Sorbus* accessions, including parental and hybrid individuals with different ploidy levels. The spectral data and the results of qualitative analyses are listed in Table 1, and the selected chromatograms are shown in Figure 1A,B. Ten compounds were identified after comparison with commercial standards: apigenin 6,8-di-C-glucoside (vicenin—2), apigenin-6-C-glucoside-8-C-arabinoside (schaftoside—4), quercetin 3-O-rutinoside (rutin—7), quercetin 3-O-galactoside (hyperoside—8), quercetin-3-O-glucoside (isoquercitrin—9), luteolin-7-O-glucoside (cynaroside—10), luteolin-7-O-glucuronide (11), kaempferol 3-O-glucoside (astragalin—16), quercetin 3-O-rhamnoide (quercitrin—17), apigenin 7-O-glucuronide (21). The structures of the other detected compounds were characterised based on similarities of the mass and UV spectra with level 3 data in the literature, according to Schymanski et al. [43].

**Table 1.** UV and MS spectral data of the detected flavonoids in EtOAc fractions of *Sorbus* accessions.

| No       | Rt (min)    | $\lambda_{max}$ (nm) | MW  | $m/z$<br>[M-H] <sup>−</sup> | Product Ions | Compound                                                   |
|----------|-------------|----------------------|-----|-----------------------------|--------------|------------------------------------------------------------|
| Flavones |             |                      |     |                             |              |                                                            |
| 2 *      | 15.07–15.94 | 270, 338             | 594 | 593                         | 226; 473     | Apigenin 6,8-di-C-glucoside (Vicenin) <sup>st</sup><br>**  |
| 4        | 18.76–19.12 | 270, 338             | 564 | 563                         | 473          | Apigenin-6-C-glucoside-8-C-arabinoside (Schaftoside)<br>st |
| 21       | 25.44–25.64 | 268, 338             | 446 | 445                         | 269          | Apigenin 7-O-glucuronide <sup>st</sup>                     |
| 10       | 22.22–22.48 | 254, 266sh, 350      | 448 | 447                         | 285          | Luteolin 7-O-glucoside (Cynaroside) <sup>st</sup>          |
| 11       | 22.47–22.74 | 254, 266sh, 350      | 462 | 461                         | 285          | Luteolin 7-O-glucuronide <sup>st</sup>                     |
| 20       | 24.78–25.08 | 248, 268sh, 340      | 448 | 447                         | 285          | Luteolin hexoside [44]                                     |

Table 1. Cont.

| No              | Rt (min)    | $\lambda_{max}$ (nm) | MW  | $m/z$<br>[M-H] <sup>-</sup> | Product Ions       | Compound                                              |
|-----------------|-------------|----------------------|-----|-----------------------------|--------------------|-------------------------------------------------------|
| Flavanols       |             |                      |     |                             |                    |                                                       |
| 1 *             | 14.99–15.12 | 252, 266sh, 372      | 626 | 625                         | 317                | Hydroxyquercetin deoxyhexosyl hexoside [45]           |
| 3               | 17.36–17.76 | 256, 264sh, 354      | 626 | 625                         | 301                | Quercetin dihexoside [23]                             |
| 5               | 19.97–20.19 | 256, 264sh, 354      | 740 | 739                         | 447; 593           | Quercetin trideoxyhexoside                            |
| 6               | 20.15–20.69 | 256, 264sh, 354      | 610 | 609                         | 301                | Quercetin deoxyhexosyl hexoside [45]                  |
| 7               | 20.40–20.73 | 256, 264sh, 354      | 610 | 609                         | 301                | Quercetin 3-O-rutinoside (Rutin) <sup>st</sup>        |
| 8               | 21.28–21.57 | 256, 264sh, 354      | 464 | 463                         | 269; 301           | Quercetin 3-O-galactoside (Hyperoside) <sup>st</sup>  |
| 9               | 21.50–21.96 | 256, 264sh, 354      | 464 | 463                         | 269; 301           | Quercetin-3-O-glucoside (Isoquercitrin) <sup>st</sup> |
| 12              | 22.67–23.10 | 266, 348             | 594 | 593                         | 285                | Kaempferol deoxyhexosylhexoside [45]                  |
| 13              | 22.61–22.96 | 256, 264sh, 354      | 596 | 595                         | 301; 433           | Quercetin hexosylpentoside [46]                       |
| 14              | 22.51–23.38 | 256, 268sh, 356      | 506 | 505                         | 301; 463           | Quercetin acetylhexoside [22,46]                      |
| 15              | 23.88–24.08 | 256, 264sh, 354      | 434 | 433                         | 301                | Quercetin pentoside [22]                              |
| 16              | 24.00–24.23 | 266, 348             | 448 | 447                         | 285                | Kaempferol 3-O-glucoside (Astragalin) <sup>st</sup>   |
| 17              | 23.98–24.30 | 256, 264sh, 354      | 448 | 447                         | 301                | Quercetin 3-O-rhamnoides (Quercitrin) <sup>st</sup>   |
| 22              | 25.80–26.27 | 266, 348             | 490 | 489                         | 285; 447           | Kaempferol acetylhexoside [46]                        |
| Methylflavonols |             |                      |     |                             |                    |                                                       |
| 18              | 23.85–24.17 | 256, 268sh, 356      | 478 | 477                         | 271; 301; 315      | Methylquercetin hexoside isomer 1 [46]                |
| 19              | 24.26–24.80 | 256, 268sh, 356      | 478 | 477                         | 271; 285; 301; 315 | Methylquercetin hexoside isomer 2 [46]                |
| 23              | 25.98–26.30 | 256, 268sh, 356      | 520 | 519                         | 285; 301; 316; 447 | Methylquercetin acetylhexoside [46]                   |

\* The numbers of the detected compounds, based on the order of elution; Rt—retention time; \*\* The references for comparison of the UV and/or MS data of the compounds are given in square brackets; st.—commercial authentic compounds used for identification; sh.—second harmonic wavelength.

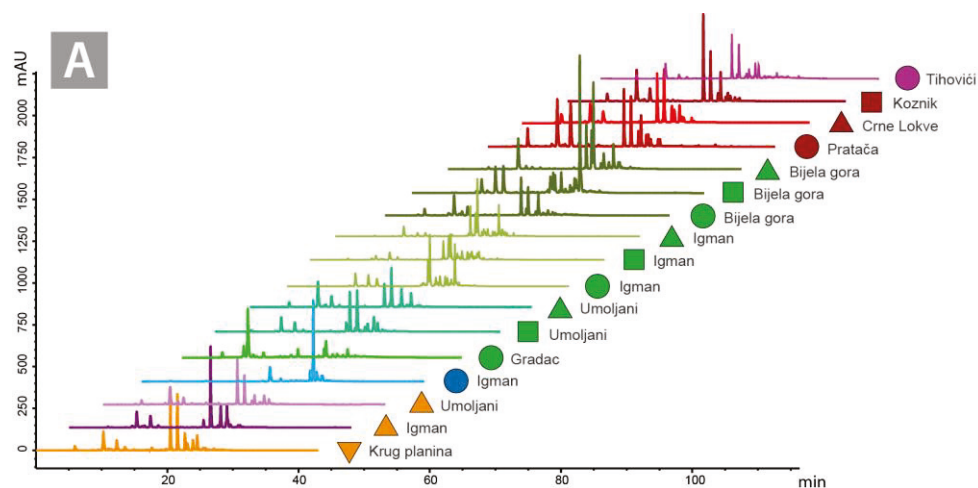
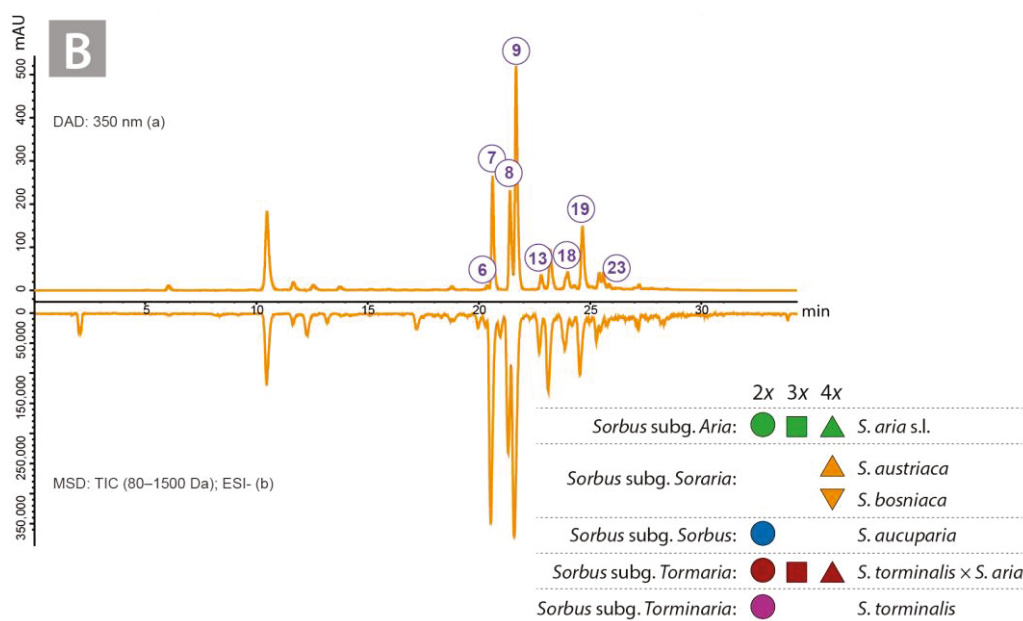


Figure 1. Cont.



**Figure 1.** Liquid chromatography–mass spectrometry chromatograms (wavelength, 350 nm) of 17 analysed ethyl acetate fractions (A). The chromatograms are supplemented with localities for each analysed accession. and liquid chromatography–mass spectrometry chromatograms (a—diode array detector, b—mass selective detector) of the ethyl acetate fraction of *Sorbus bosniaca* leaves (B). Compound numbers are in circles and correspond to those in Table 1. The chromatograms are supplemented with localities for each analysed accession.

### 2.2.1. Flavones

Compounds **2**, **4** and **21** were assigned to apigenin glycosides based on their UV spectra (Table 1). The mass spectra (MS) of apigenin 7-*O*-glucuronide (**21**; [M−H]<sup>−</sup> at *m/z* 445) contained signal corresponding to the aglycone at *m/z* 269 formed by the neutral loss of 176 Da, whereas flavonoids **2** and **4** showed different fragmentation patterns of C-flavonoid glycosides ([M−H]<sup>−</sup> at *m/z* 593, and 563, respectively). As the C–C glycosidic bonds are more stable than *O*-glycosidic bonds, those flavonoids cannot yield fragments due to the loss of neutral sugar, but fragments resulting from the breakdown of the C–C bonds within the sugar moiety and total cross-ring cleavage [47,48]. In all three cases, the reference compounds confirmed the identity. The UV and MS spectra of **10**, **11** and **20** corresponded to luteolin glycosides (the MS spectra contained a fragment ion at *m/z* 285 corresponding to the aglycone). Compounds **10** and **20** showed a luteolin hexoside ([M−H]<sup>−</sup> at *m/z* 447, fragment ion at 285), whereas **11** had a luteolin hexuronide structure ([M−H]<sup>−</sup> at *m/z* 461, fragment ion at 285). The compounds identified as luteolin 7-*O*-glucoside (**10**) and luteolin 7-*O*-glucuronide (**11**) were confirmed with standard compounds.

### 2.2.2. Flavonols

The UV spectra of flavonoids **1**, **3**, **5–9**, **12–15**, **16**, **17**, and **22** indicated the 3-substituted flavonol structure [49]. All mentioned flavonoids (except **12**, **16** and **22**) had MS spectra containing the signal of deprotonated quercetin molecule at *m/z* 301 (formed after sugar losses), and fragment ions from its RDA reactions [50]. Quercetin 3-*O*-rutinoside (**7**; [M−H]<sup>−</sup> at *m/z* 609), quercetin 3-*O*-galactoside (**8**; [M−H]<sup>−</sup> at *m/z* 463), quercetin 3-*O*-glucoside (**9**; [M−H]<sup>−</sup> at *m/z* 463), and quercetin 3-*O*-rhamnoside (**17**) with the signal of the deprotonated molecule at *m/z* 447 were identified from standard compounds. Additional quercetin diglycosides **3**, **6** and **13** showed fragmentation patterns corresponding to quercetin dihexoside (**3**; [M−H]<sup>−</sup> at *m/z* 625), quercetin deoxyhexosyl hexoside (**6**; [M−H]<sup>−</sup> at *m/z* 609),

and quercetin hexosylpentoside (**13**;  $[M-H]^-$  at  $m/z$  595), respectively. The fragment ions were generated by the loss of one or more hexose units (162 Da), deoxyhexose (146 Da), or pentose moiety (132 Da).

The structure of compound **5** was assigned to quercetin trideoxyhexoside, while **14** was characterised as quercetin acetylhexoside (with signals of  $[M-H]^-$  at  $m/z$  505, fragment ions at  $m/z$  463 and 301), and **15** as quercetin pentoside ( $[M-H]^-$  at  $m/z$  433, fragment ion at  $m/z$  301). The kaempferol glycosides (**12**, **16**, **22**) were identified from the standard compound in the case of kaempferol 3-*O*-glucoside (**16**;  $[M-H]^-$  at  $m/z$  447), or by analyzing their mass spectra. Therefore, **12** was characterised as kaempferol deoxyhexosylhexoside ( $[M-H]^-$  at  $m/z$  593, fragment ion at  $m/z$  285), and **22** ( $[M-H]^-$  at  $m/z$  489) as acetylhexoside of kaempferol, based on the neutral loss of 42 Da corresponding to the acetyl group and the loss of hexose.

### 2.2.3. Methylated Flavonols

Flavonoid glycosides **18**, **19** and **23** ( $[M-H]^-$  at  $m/z$  477 and 519) showed mass spectra related to threehydroxy-methoxy-flavone-3-ol with fragment ions at  $m/z$  316 (corresponding to aglycone) originating from neutral losses of hexose (in **18** and **19**) and acetylhexose (**23**); 301 (additional neutral loss of methyl group), 285 (additional loss of methoxy group).

### 2.3. Quantitative Analysis of Flavonoids

The quantification of the 10 identified flavonoids was performed using the external standard method with authentic commercial standards. Due to structural similarities, five of these standards were also used for the quantification of the remaining 13 identified flavonoids. The regression equations of the calibration curves, their correlation coefficients ( $r^2$ ), concentration ranges, LODs and LOQs are shown in Table S1. Secondary metabolites present in only one individual were not considered in the analysis. The amounts of flavonoids identified (in g/100 g DE) are listed in Table 2. Analysis of the ethyl acetate fractions revealed that quercetin flavonoids were the predominant component of the flavonoid profile in almost all studied *Sorbus* accessions (accounting for up to 75.34% of the total flavonoids detected in triploid *S. aria* from Mt. Igman). Luteolin flavonoids and apigenin flavonoids were detected only in samples of the subg. *Tormaria* group accounted for 6.5–17.8% and 1.7–60% of the total flavonoids identified, respectively.

Kaempferol flavonoids ranged between 1.3 and 21.8% and 2.0 and 3.5% of the total flavonoids detected in the subg. *Soraria* and *Tormaria*, respectively. Within the subg. *Soraria* group, a single triploid cytotype of *S. aria* from Umoljani is characterised by the trace/absence of kaempferol derivatives (0% for compounds **21** and **25** and a trace amount for compound **33**). Within the *Tormaria* group, a single triploid cytotype of *S. torminalis* × *S. aria* from Koznik was characterised by the complete absence of kaempferol derivatives.

**Table 2.** Quantities (% g/100 g DE) of detected flavonoids in EtOAc fractions of *Sorbus* accessions.

| Taxon                              | Subgenus                                            | Location                 | DNA<br>Ploidy<br>Level | Flavones |       |         |     |         |       |         |       |         |       |         |
|------------------------------------|-----------------------------------------------------|--------------------------|------------------------|----------|-------|---------|-----|---------|-------|---------|-------|---------|-------|---------|
|                                    |                                                     |                          |                        | Xsr (%)  | STD   | Xsr (%) | STD | Xsr (%) | STD   | Xsr (%) | STD   | Xsr (%) | STD   |         |
|                                    |                                                     |                          |                        | 2        |       | 4       |     | 21      |       | 10      |       | 11      |       | 20      |
| <i>S. aria</i>                     | <i>Aria</i>                                         | Grkarica, Mt. Igman      | 2x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Bijela gora, Mt. Orjen   | 2x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Gradac, Posušje          | 2x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Grkarica, Mt. Igman      | 3x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Umoljani, Mt. Bjelašnica | 3x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Bijela gora, Mt. Orjen   | 3x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Grkarica, Mt. Igman      | 4x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Umoljani, Mt. Bjelašnica | 4x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Bijela gora, Mt. Orjen   | 4x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Veliko polje, Mt. Igman  | 2x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
| <i>S. aucuparia</i>                | <i>Sorbus</i>                                       |                          |                        |          |       |         |     |         |       |         |       |         |       |         |
| <i>S. austriaca</i>                | <i>Soraria</i> ( <i>Sorbus</i> × <i>Aria</i> )      | Grkarica, Mt. Igman      | 4x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
|                                    |                                                     | Umoljani, Mt. Bjelašnica | 4x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
| <i>S. bosniaca</i>                 |                                                     | Mt. Krug planina         | 4x                     | n.d.     |       | n.d.    |     | n.d.    |       | n.d.    |       | n.d.    |       | n.d.    |
| <i>S. torminalis</i> × <i>aria</i> | <i>Tormaria</i> ( <i>Torminalis</i> × <i>Aria</i> ) | Pratača, Lokve           | 2x                     | 0.226 *  | 0.015 | trace * | /   | 2.414 * | 0.013 | 0.107 * | 0.013 | 0.464 * | 0.005 | 0.161 * |
|                                    |                                                     | Koznik                   | 3x                     | 0.059 *  | 0.014 | n.d.    |     | 0.617 * | 0.092 | 0.148 * | 0.012 | 0.348 * | 0.006 | 0.007 * |
|                                    |                                                     | Crne lokve, Posušje      | 4x                     | 0.167 *  | 0.015 | trace * | /   | n.d.    |       | 0.206 * | 0.014 | 0.347 * | 0.005 | 1.224 * |
| <i>S. torminalis</i>               | <i>Torminaria</i>                                   | Tihovići                 | 2x                     | n.d.     |       | trace * | /   | 8.913   | 0.124 | 0.186 * | 0.013 | 1.159 * | 0.020 | 0.506 * |
|                                    |                                                     |                          |                        |          |       |         |     |         |       |         |       |         |       | 0.007   |



| Taxon                                 | Subgenus                                               | Location                 | DNA<br>Ploidy<br>Level | Flavonols  |       |            |            |            |       |            |       |            |       |            |       |  |
|---------------------------------------|--------------------------------------------------------|--------------------------|------------------------|------------|-------|------------|------------|------------|-------|------------|-------|------------|-------|------------|-------|--|
|                                       |                                                        |                          |                        | Xsr<br>(%) | STD   | Xsr<br>(%) | STD        | Xsr<br>(%) | STD   | Xsr<br>(%) | STD   | Xsr<br>(%) | STD   | Xsr<br>(%) | STD   |  |
|                                       |                                                        |                          |                        | 1          | 3     | 5          | 6          | 7          | 8     | 9          |       |            |       |            |       |  |
| <i>S. aria</i>                        | <i>Aria</i>                                            | Grkarica, Mt. Igman      | 2x                     | n.d.       | n.d.  | n.d.       | n.d.       | 4.738      | 0.022 | n.d.       | n.d.  | 1.779      | 0.011 |            |       |  |
|                                       |                                                        | Mt. Bijela gora          | 2x                     | n.d.       | n.d.  | n.d.       | n.d.       | 0.577      | 0.012 | n.d.       | n.d.  | 2.847      | 0.005 |            |       |  |
|                                       |                                                        | Gradac, Posušje          | 2x                     | n.d.       | n.d.  | n.d.       | trace<br>* | 1.127      | 0.012 | 0.568      | 0.006 | 3.738      | 0.005 |            |       |  |
|                                       |                                                        | Grkarica, Mt. Igman      | 3x                     | n.d.       | n.d.  | n.d.       | n.d.       | 7.504      | 0.019 | n.d.       | n.d.  | 1.791      | 0.007 |            |       |  |
|                                       |                                                        | Umoljani, Mt. Bjelašnica | 3x                     | n.d.       | n.d.  | 0.978<br>* | 0.003      | 3.555      | 0.008 | 0.249      | 0.007 | 1.586      | 0.003 |            |       |  |
|                                       |                                                        | Mt. Bijela gora          | 3x                     | 0.083<br>* | 0.005 | n.d.       | n.d.       | 3.715      | 0.027 | 0.305      | 0.008 | 1.266      | 0.014 |            |       |  |
| <i>S. aucuparia</i>                   | <i>Sorbus</i>                                          | Grkarica, Mt. Igman      | 4x                     | n.d.       | n.d.  | n.d.       | n.d.       | 4.115      | 0.044 | 0.138      | 0.005 | 1.898      | 0.016 |            |       |  |
|                                       |                                                        | Umoljani, Mt. Bjelašnica | 4x                     | n.d.       | n.d.  | n.d.       | n.d.       | 4.115      | 0.044 | 0.138      | 0.005 | 1.898      | 0.016 |            |       |  |
|                                       |                                                        | Mt. Bijela gora          | 4x                     | n.d.       | n.d.  | n.d.       | n.d.       | 5.330      | 0.010 | n.d.       | n.d.  | 2.004      | 0.007 |            |       |  |
|                                       |                                                        | Veliko polje, Mt. Igman  | 2x                     | n.d.       | 0.346 | 0.004      | n.d.       | 0.196      | 0.013 | 0.460      | 0.014 | 0.641      | 0.004 |            |       |  |
| <i>S. austriaca</i>                   | <i>Soraria</i><br>( <i>Sorbus</i> × <i>Aria</i> )      | Grkarica, Mt. Igman      | 4x                     | 0.377<br>* | 0.009 | n.d.       | trace<br>* | 3.175      | 0.023 | 0.469      | 0.015 | 1.043      | 0.008 |            |       |  |
| <i>S. bosniaca</i>                    |                                                        | Umoljani, Mt. Bjelašnica | 4x                     | n.d.       | trace | trace<br>* | /          | 1.960      | 0.029 | 0.223      | 0.011 | 1.345      | 0.017 |            |       |  |
|                                       |                                                        | Mt. Krug planina         | 4x                     | n.d.       | n.d.  | n.d.       | 0.179<br>* | 3.695<br>* | 0.020 | 2.658<br>* | 0.012 | 3.102<br>* | 0.018 |            |       |  |
| <i>S. torminalis</i><br>× <i>aria</i> | <i>Tormaria</i><br>( <i>Torminalis</i> × <i>Aria</i> ) | Pratača, Mt. Igman       | 2x                     | n.d.       | n.d.  | n.d.       | 0.223      | 0.010      | 0.267 | 0.021      | 1.896 | 0.014      | 1.883 | 0.011      |       |  |
|                                       |                                                        | Koznik                   | 3x                     | n.d.       | n.d.  | trace      | /          | 0.098      | 0.003 | 1.594      | 0.020 | 1.592      | 0.014 | 0.939      | 0.009 |  |
|                                       |                                                        | Crne lokve, Posušje      | 4x                     | n.d.       | n.d.  | n.d.       | n.d.       | 0.265      | 0.008 | 2.520      | 0.009 | 1.613      | 0.009 | 2.063      | 0.003 |  |
| <i>S. torminalis</i>                  | <i>Torminaria</i>                                      | Tihovići                 | 2x                     | n.d.       | n.d.  | n.d.       | n.d.       | 0.236      | 0.010 | 1.284      | 0.009 | 0.642      | 0.01  |            |       |  |

Table 2. Cont.

| Taxon                              | Subgenus                                               | Location                 | DNA Ploidy Level | Flavonols |         |         |       |         |         |         |       |         |         |
|------------------------------------|--------------------------------------------------------|--------------------------|------------------|-----------|---------|---------|-------|---------|---------|---------|-------|---------|---------|
|                                    |                                                        |                          |                  | Xsr (%)   | STD     | Xsr (%) | STD   | Xsr (%) | STD     | Xsr (%) | STD   | Xsr (%) | STD     |
|                                    |                                                        |                          |                  | 12        | 13      | 14      | 15    | 16      | 17      | 22      |       |         |         |
| <i>S. aria</i>                     | <i>Aria</i>                                            | Grkarica, Mt. Igman      | 2x               | 0.886 *   | 0.005   | n.d.    | 2.582 | 0.015   | n.d.    | 0.698   | 0.005 | n.d.    | 0.366 * |
|                                    |                                                        | Mt. Bijela gora          | 2x               | n.d.      | 0.167 * | 0.01    | n.d.  | 0.003   | 0.010   | n.d.    | 2.736 | 0.010   | 0.108 * |
|                                    |                                                        | Gradac, Posušje          | 2x               | n.d.      | 0.131 * | 0.007   | 0.650 | 0.003   | 0.016   | 0.308   | 0.016 | n.d.    | n.d.    |
|                                    |                                                        | Grkarica, Mt. Igman      | 3x               | 0.459 *   | 0.002   | n.d.    | 2.380 | 0.010   | n.d.    | 0.263   | 0.003 | n.d.    | 0.244 * |
|                                    |                                                        | Umoljani, Mt. Bjelašnica | 3x               | n.d.      | 0.435 * | 0.010   | 0.783 | 0.004   | n.d.    | n.d.    | 1.853 | 0.003   | trace * |
|                                    |                                                        | Mt. Bijela gora          | 3x               | 0.330 *   | 0.048   | 0.500 * | 1.047 | 0.132   | n.d.    | n.d.    | 1.651 | 0.000   | n.d.    |
|                                    |                                                        | Grkarica, Mt. Igman      | 4x               | 0.958 *   | 0.012   | n.d.    | 1.323 | 0.031   | n.d.    | 1.056   | 0.015 | n.d.    | 0.231 * |
|                                    |                                                        | Umoljani, Mt. Bjelašnica | 4x               | 0.209 *   | 0.007   | n.d.    | 0.774 | 0.013   | n.d.    | 0.136   | 0.006 | n.d.    | trace * |
|                                    |                                                        | Mt. Bijela gora          | 4x               | 1.033 *   | 0.015   | n.d.    | 0.668 | 0.009   | n.d.    | 0.624   | 0.008 | n.d.    | trace * |
|                                    |                                                        | Veliko polje, Mt. Igman  | 2x               | 0.288     | 0.022   | n.d.    | 0.331 | 0.005   | n.d.    | n.d.    | n.d.  | n.d.    | trace   |
| <i>S. austriaca</i>                | <i>Soraria</i><br>( <i>Sorbus</i> × <i>Aria</i> )      | Grkarica, Mt. Igman      | 4x               | n.d.      | 0.281 * | 0.006   | 2.103 | 0.021   | 0.384 * | n.d.    | 0.003 | n.d.    | 0.153 * |
|                                    |                                                        | Umoljani, Mt. Bjelašnica | 4x               | n.d.      | 0.128 * | 0.003   | 1.609 | 0.012   | 0.222 * | n.d.    | 0.015 | n.d.    | 0.082 * |
|                                    |                                                        | Mt. Krug planina         | 4x               | n.d.      | 0.590 * | 0.01    | n.d.  | n.d.    | n.d.    | n.d.    | n.d.  | n.d.    | 0.152 * |
| <i>S. torminalis</i> × <i>aria</i> | <i>Tornaria</i><br>( <i>Torminalis</i> × <i>Aria</i> ) | Pratača, Mt. Igman       | 2x               | n.d.      | n.d.    | 0.367   | 0.013 | 0.006   | n.d.    | n.d.    | 0.006 | n.d.    | 0.198   |
|                                    |                                                        | Koznik                   | 3x               | n.d.      | 0.353   | 0.780   | 0.010 | 0.010   | n.d.    | n.d.    | 0.010 | n.d.    | n.d.    |
|                                    |                                                        | Crne lokve, Posušje      | 4x               | 0.346     | 0.039   | 0.431   | 0.004 | 0.004   | n.d.    | n.d.    | 0.004 | n.d.    | n.d.    |
| <i>S. torminalis</i>               | <i>Torminaria</i>                                      | Tihovići                 | 2x               | n.d.      | n.d.    | n.d.    | n.d.  | 0.339   | 0.008   | n.d.    | n.d.  | n.d.    | n.d.    |

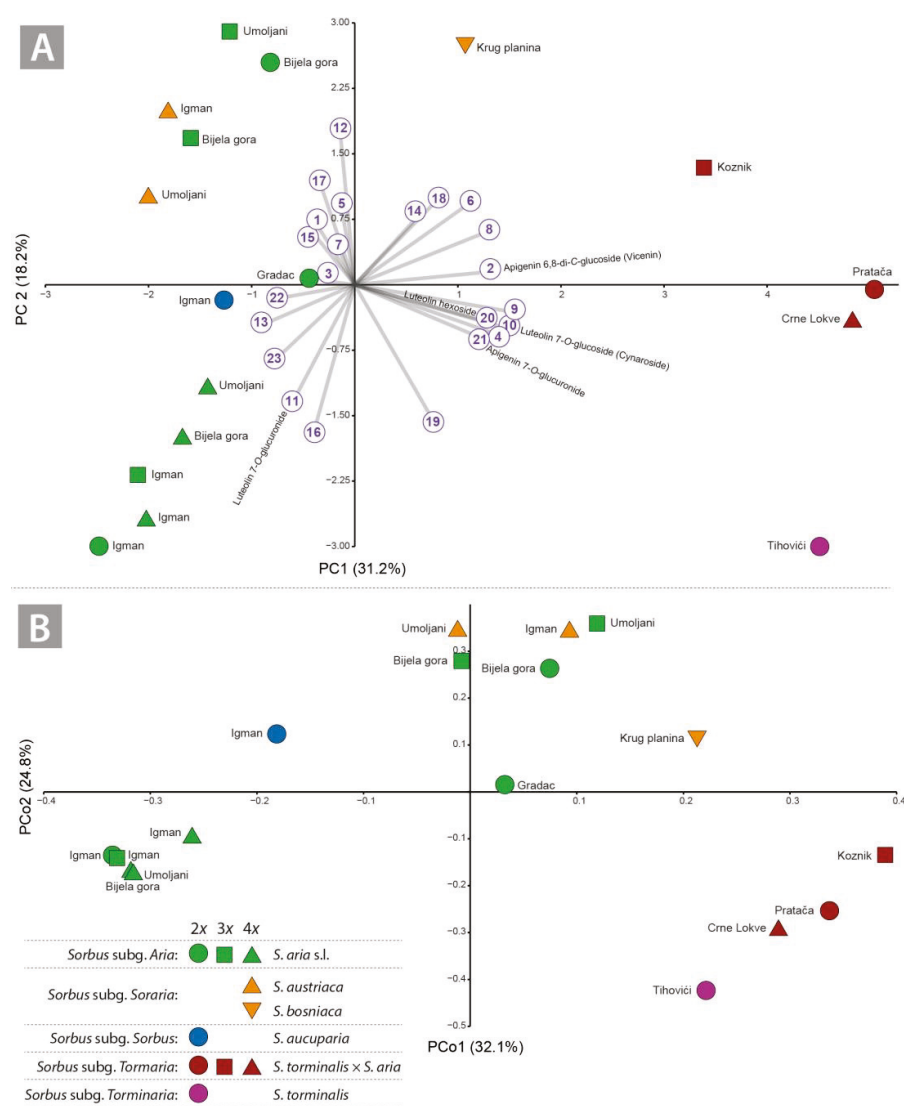
Table 2. Cont.

| Taxon                              | Subgenus                                            | Location                 | DNA Ploidy Level | Methylflavonols      |              |                      |              |                      |              |
|------------------------------------|-----------------------------------------------------|--------------------------|------------------|----------------------|--------------|----------------------|--------------|----------------------|--------------|
|                                    |                                                     |                          |                  | X <sub>s</sub> r (%) | STD          | X <sub>s</sub> r (%) | STD          | X <sub>s</sub> r (%) | STD          |
|                                    |                                                     |                          |                  | <b>18</b>            |              | <b>19</b>            |              | <b>23</b>            |              |
| <i>S. aria</i>                     | <i>Aria</i>                                         | Grkarica, Mt. Igman      | 2x               | n.d.                 |              | <b>1.016 *</b>       | <b>0.006</b> | <b>0.444 *</b>       | <b>0.005</b> |
|                                    |                                                     | Mt. Bijela gora          | 2x               | n.d.                 |              | n.d.                 |              | <b>0.134 *</b>       | <b>0.002</b> |
|                                    |                                                     | Gradac, Posušje          | 2x               | n.d.                 |              | <b>0.476 *</b>       | <b>0.009</b> | n.d.                 |              |
|                                    |                                                     | Grkarica, Mt. Igman      | 3x               | n.d.                 |              | <b>0.724 *</b>       | <b>0.003</b> | <b>0.205 *</b>       | <b>0.007</b> |
|                                    |                                                     | Umoljani, Mt. Bjelašnica | 3x               | n.d.                 |              | n.d.                 |              | n.d.                 |              |
|                                    |                                                     | Mt. Bijela gora          | 3x               | n.d.                 |              | n.d.                 |              | n.d.                 |              |
|                                    |                                                     | Grkarica, Mt. Igman      | 4x               | n.d.                 |              | <b>0.496 *</b>       | <b>0.021</b> | <b>0.087 *</b>       | <b>0.008</b> |
|                                    |                                                     | Umoljani, Mt. Bjelašnica | 4x               | n.d.                 |              | <b>0.172 *</b>       | <b>0.002</b> | n.d.                 |              |
|                                    |                                                     | Mt. Bijela gora          | 4x               | n.d.                 |              | <b>0.173 *</b>       | <b>0.003</b> | n.d.                 |              |
| <i>S. aucuparia</i>                | <i>Sorbus</i>                                       | Veliko polje, Mt. Igman  | 2x               | n.d.                 |              | n.d.                 |              | n.d.                 |              |
| <i>S. austriaca</i>                | <i>Soraria</i> ( <i>Sorbus</i> × <i>Aria</i> )      | Grkarica, Mt. Igman      | 4x               | n.d.                 |              | n.d.                 |              | n.d.                 |              |
|                                    |                                                     | Umoljani, Mt. Bjelašnica | 4x               | n.d.                 |              | n.d.                 |              | <b>trace *</b>       | /            |
| <i>S. bosniaca</i>                 |                                                     | Mt. Krug planina         | 4x               | <b>0.881 *</b>       | <b>0.007</b> | <b>0.186 *</b>       | <b>0.003</b> | n.d.                 |              |
| <i>S. torminalis</i> × <i>aria</i> | <i>Tornaria</i> ( <i>Torminalis</i> × <i>Aria</i> ) | Pratača, Mt. Igman       | 2x               | 0.833                | 0.014        | 0.738                | 0.014        | n.d.                 |              |
|                                    |                                                     | Koznik                   | 3x               | 0.710                | 0.008        | 0.525                | 0.021        | n.d.                 |              |
|                                    |                                                     | Crme lokve, Posušje      | 4x               | 0.427                | 0.008        | 0.386                | 0.007        | n.d.                 |              |
| <i>S. torminalis</i>               | <i>Torminaria</i>                                   | Tihovići                 | 2x               | n.d.                 |              | 1.669                | 0.015        | n.d.                 |              |

X<sub>s</sub>r—mean value of triplicates; STD—standard deviation; DE—dry extract. The names of the secondary metabolites correspond to their numerical values, which are given by Table 1: UV and MS spectral data of the detected flavonoids in EtOAc fractions of *Sorbus* accessions. Newly identified compounds from the leaves of *Sorbus* accessions are highlighted using bold font and marked with asterisk (\*).

#### 2.4. Pattern of Flavonoid Variation and Relationships Among the Studied Sorbus Samples

Phytochemical variation based on the quantitative data was complex and principal component analysis revealed eight significant principal components (Eigenvalues  $\geq 1$ ) (two components are shown). They accounted for 49.3% of the total variance (PC1 = 31.2%, PC2 = 18.1%; Figure 2A) and showed moderate correlation with most of the corresponding flavonoid compounds (Table S2). The following compounds contributed most strongly to PC1 (schaftoside, isoquercitrin, luteolin 7-O-glucoside and luteolin hexoside; luteolin 7-O-glucuronide, kaempferol deoxyhexosylhexoside, astragalin and methylquercetin hexoside isomer 2 contributed to PC2 (Table S2). The PCA ordination diagram showed the pattern in which *S. torminalis* and all *Tormaria* representatives along PC1 clearly diverged from the other *Sorbus* accessions (Figure 2A). The position of *S. bosniaca* was also divergent (Figure 2A). Along PC2, *S. austriaca* overlapped with a single diploid and two triploid *S. aria* samples separated from *S. aucuparia* and the remaining of *S. aria* samples, including all tetraploids (Figure 2A).



**Figure 2.** PCA and PCoA ordination ((A)—PC1 vs. PC2; (B)—PCo1 vs. PCo2) of the flavonoid compounds of the investigated *Sorbus* accessions. The ordination diagrams are supplemented with the localities for each studied accession. Compound numbers in circles (A) correspond to those in Table 1.

The PCoA analysis of all individuals showed a similar pattern as the PCA but with a higher resolution among the studied accessions (Figure 2B). *Sorbus torminalis* and all *Tormaria* members, as well as the diploids (Bijela gora and Gradac) and triploids (Bijela gora and Umoljani) of *S. aria*, *S. austriaca* and *S. bosniaca* are clearly distinct along PCo1 from *S. aucuparia* and all tetraploids of *S. aria* as well as the diploids and triploids of Mt. Igman. *Sorbus aucuparia* was separated from different *S. aria* cytotypes along PCo2 (Figure 2B). The members of the *Soraria* subg. (*S. austriaca* and *S. bosniaca*) and the cytotypes of *S. aria* were separated along PCo2 from the members of *S. torminalis* and *Tormaria* (Figure 2B).

PCoA ordination of *Tormaria* members and putative parental species showed a clear position of *S. torminalis* and *Tormaria* cytotypes compared to *S. aria* cytotypes (Figure 3A). The cytotypes of *S. torminalis* × *S. aria* were clearly closer to *S. torminalis* than to *S. aria* (Figure 3A).

The ordination of *Soraria* samples (*S. austriaca* and *S. bosniaca*) showed an interesting pattern of spatial distribution between PCo1 and PCo2 (Figure 3B). Along PCo1, *S. bosniaca* and the diploid *S. aria* from Bijela gora diverged the most; the triploids of *S. aria* (Umoljani and Bijela gora) and the diploid *S. aria* from Bijela gora intermingled with *S. austriaca* compared to the tetraploids of *S. aucuparia* and *S. aria* and the diploids and triploids from Mt. Igman (Figure 3B). *Sorbus aucuparia* had an intermediate position along the PCo2 compared to the di-, tri- and tetraploid cytotypes of *S. aria* and *S. austriaca*. Along PCo2, *S. bosniaca* was clearly separated from the cytotypes of *S. austriaca* and *S. aria* (Figure 3B).

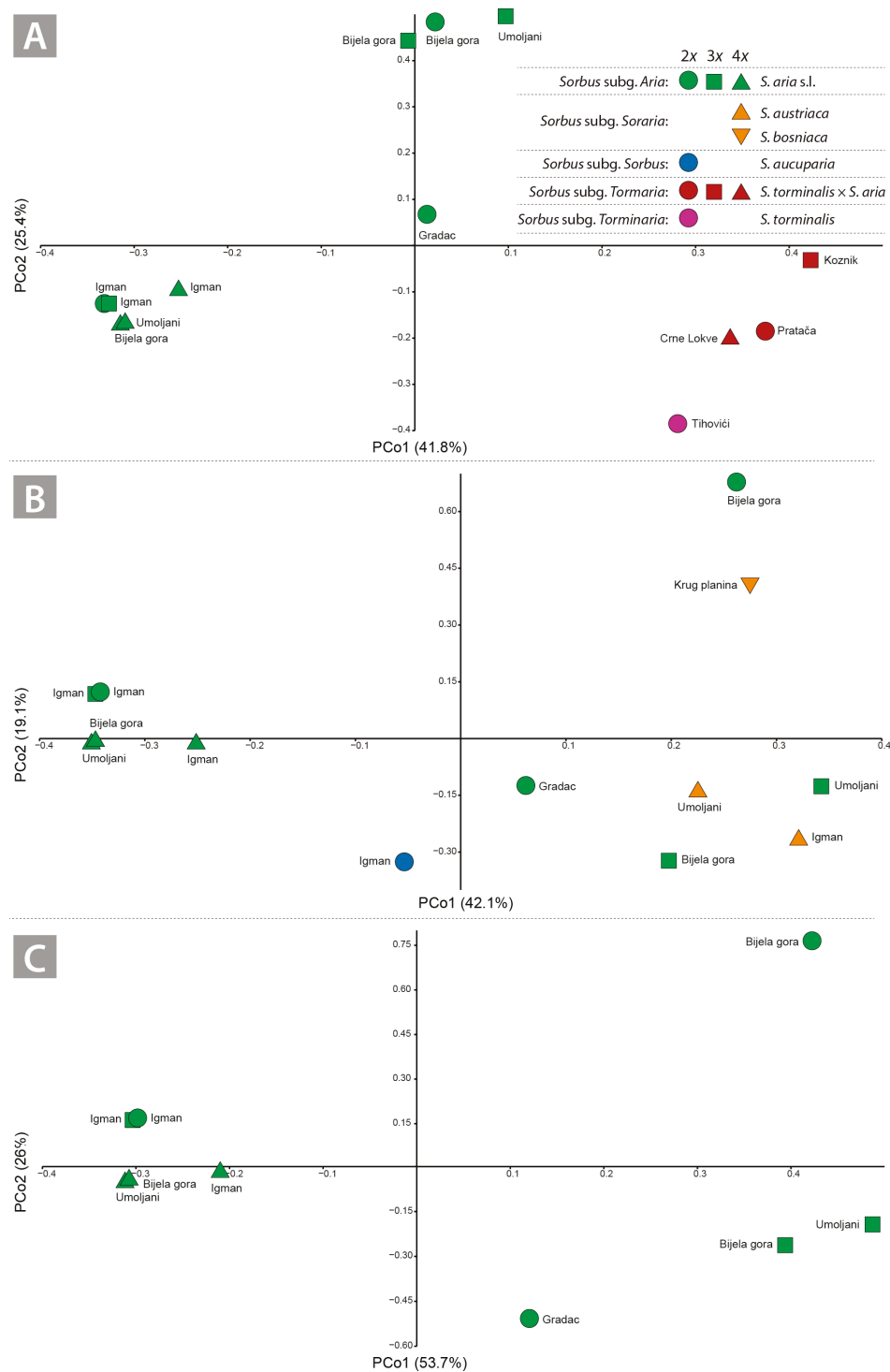
The most intriguing pattern was observed in the cytotypes of the *S. aria* (Figure 3C). The diploids of *S. aria* were the most divergent and separated along PCo1 and PCo2 (Figure 3C). While the tetraploid *S. aria* cytotypes represented a uniform group, the triploids segregated along PCo1. No specific pattern was recognisable for the *S. aria* cytotypes.

The UPGMA cluster analysis revealed two main clusters (Figure 4A). The first cluster contained *S. torminalis* and its hybrids *S. torminalis* × *S. aria*. The second cluster contained two subclusters, one of which contained *S. bosniaca*, *S. austriaca*, *S. aria* diploids (Bijela gora and Gradac) and triploids (Umoljani and Bijela gora), while the second contained *S. aucuparia* and a group consisting of exclusively *S. aria* samples (Figure 4A).

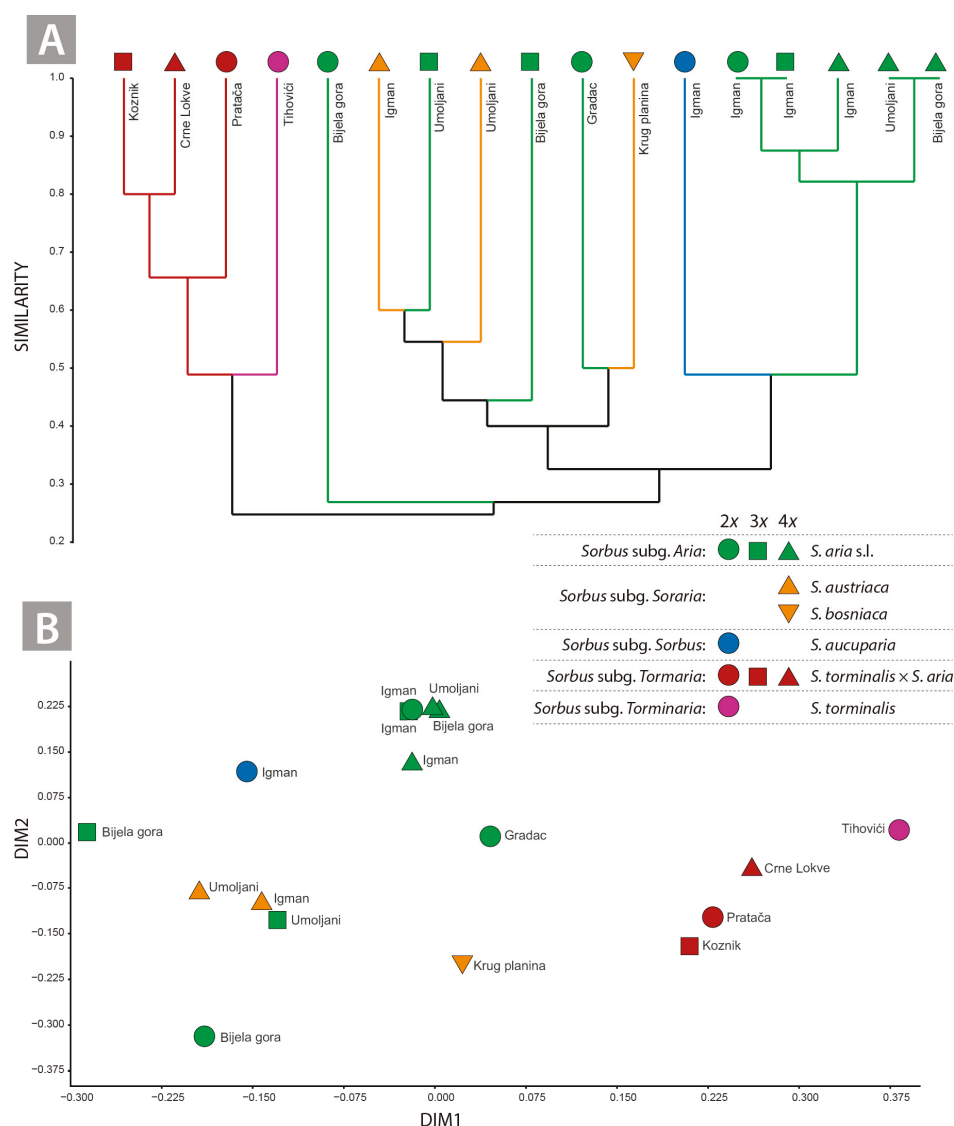
The NMDS plot was consistent with the results of the previous multivariate analyses but showed a clearer separation between the *Sorbus* samples analysed (stress value  $S = 0.202$  (Figure 4B). The relatively moderate stress value ( $S = 0.202$ ) among the flavonoid compounds in the original data matrix is well illustrated in the ordination diagram (Figure 3B). The samples of *Sorbus torminalis*, *S. aucuparia*, *S. bosniaca*, the diploid *S. aria* from Bijela gora and the hybrid *S. torminalis* × *S. aria* differed significantly from each other. Di-, tri- and tetraploid *S. aria* cytotypes from Mt. Igman, Bijela gora and Umoljani were grouped together, while *S. austriaca* overlapped with the triploids *S. aria* (Figure 4B). Both the qualitative and quantitative values of the luteolin and apigenin derivatives clearly distinguished the *Tormaria* group, especially between the parental species *S. torminalis* and the closest cluster, which comprised hybrid derivatives of *S. torminalis* × *S. aria*. The active components that grouped di, tri and tetraploid individuals of *S. aria* from Mt. Igman, Bijela and Umoljani into a unique cluster were quercetin and kaempferol derivatives. In the differentiation of *S. aucuparia*, *S. bosniaca*, the diploid *S. aria* from Bijela gora, *S. austriaca* and the triploids of *S. aria*, predominantly quercetin derivatives were detected as discriminating metabolites.

The results of the Mantel test showed a weak and significant correlation between the Bray–Curtis distances ( $r = 0.285$ ,  $p \leq 0.004$ ) for all studied accessions. In contrast, no correlation was found for the members of the subgenus *Aria* ( $r = 0.044$ ,  $p \leq 0.354$ ).





**Figure 3.** PCoA ordination (PCo1 vs. PCo2) of the flavonoid compounds of the investigated *Sorbus* accessions given for the subgenera *Tormaria* (A), *Soraria* (B), and *Aria* (C). The ordination diagrams are supplemented with the localities for each studied accession.



**Figure 4.** Results of multivariate analyses for 17 analysed *Sorbus* accessions. (A)—dendrogram showing the flavonoid relationships between the flavonoids of the accessions using the UPGMA method; (B)—non-metric dimensional scaling analysis of the studied accessions. Each figure is supplemented with localities for studied accessions.

### 3. Discussion

#### 3.1. Novel Flavonoid Compounds in the Leaves of *Sorbus* Accessions

Our study reports the detection of 23 flavonoids in the EtOAc fractions from methanol (MeOH) leaf extracts of all *Sorbus* accessions examined, including 14 quercetin derivatives and three derivatives of apigenin, luteolin and kaempferol. This study is the first to analyse samples of triploid and tetraploid *Tormaria* accessions, *S. bosniaca*, and diploid and polyploid cytotypes of *S. aria* from the Balkan region. From the point of view of phytochemical research, this study demonstrated for the first time the presence of 14 flavonoids (Table 2) that had not been previously detected in the leaves of the *Sorbus* accessions studied. Among them, the rare flavonoids C-glycosides (apigenin 6,8-di-C-glucoside, apigenin-6-C-glucoside-8-C-arabinoside) were identified for the first time in representatives of the subg. *Tormaria*. Hydroxyquercetin deoxyhexosyl hexoside (1), quercetin trideoxyhexoside (5), quercetin deoxyhexosyl hexoside (6), quercetin hexosylpentoside (13), methylquercetin acetylhexoside (23), kaempferol deoxyhexosylhexoside (12) and kaempferol acetylhexoside

(20) were found for the first time in *S. aria* and *S. austriaca*, while methylquercetin hexoside isomer 2 (19) was found exclusively in *S. aria* and quercetin pentoside in *S. austriaca*. For the first time, the presence of luteolin-7-O-glucoside (10), luteolin-7-O-glucuronide (11), luteolin hexoside (20) and methylquercetin hexoside isomer 2 (19) was detected in leaves of *S. torminalis*. In addition, this study is the first to show the flavonoid profile of leaves of different *S. torminalis* × *S. aria* cytotypes from the *Tormaria* subg. as well as the endemic species *S. bosniaca* from the *Soraria* subg. (Table 2).

Compared to the EtOAc fractions of *S. aria* leaves from the cultivars analysed in the study by Olszewska and Michael [51], the EtOAc fractions of *S. aria* leaves from the Bosnia and Herzegovina region contained higher amounts of the active components hyperoside (trace amounts in the study by Olszewska and Michael [51], up to  $0.568 \pm 0.006$  g/100 g in our analysed dry extract) and rutin ( $1.37 \pm 0.03$  g/100 g in the samples reported by Olszewska and Michael [51], up to  $7.504 \pm 0.019$  g/100 g in our analysed samples). On the other hand, the analysed EtOAc fractions contained lower amounts of astragalin and isoquercitrin compared to the EtOAc fractions of the cultivars from the study by Olszewska and Michael [51]. The EtOAc fractions of *S. torminalis* leaves from the natural habitat and the EtOAc fractions of *S. torminalis* leaves from Bosnia and Herzegovina analysed by Gunes AK et al. [21] are characterised by a high concentration of hyperoside ( $1.226 \pm 0.014$  g/100 g DE and  $1.284 \pm 0.009$  g/100 g DE, respectively). Although quercitrin was not detected in the EtOAc fractions of *S. torminalis* analysed in our study, it was identified in the fractions analysed in the study by Gunes AK et al. [21]. In our study, methylquercetin hexoside isomer 2 (19) was detected for the first time in the EtOAc fraction of *S. torminalis* leaves at a concentration of  $1.669 \pm 0.015$  g/100 g dry extract (DE).

### 3.2. Potential Discrimination of Subgenera and Lower Taxa in Sorbus: Influence of Flavonoid Profile on Hybridisation and Ploidy Level

Our analysis based on flavonoid profiles revealed intriguing relationships between the *Sorbus* accessions studied. Analyses based on quantitative data (PCA) and analyses based on binary data (PCoA, CA and NDMS) generally showed a similar pattern, although the latter provided a clearer resolution. The discrimination of the parental species *S. aucuparia* and *S. torminalis* is clear, in contrast to *S. aria*, which showed a remarkable divergence within the intragroup regardless of ploidy level and geographical origin (Figure 2A,B). The discrimination of subg. *Aria* and the hybrid subg. *Tormaria* and *Soraria* showed different patterns. While the members of subg. *Tormaria* are clearly diverge from each other and are more related to *S. torminalis* (Figure 2A,B and Figure 3A), the relationships in subg. *Aria* and *Soraria* are less clear (Figure 2A,B and Figure 3B,C). Indeed, the number of distinctive flavonoids detected in *Tormaria* was notably higher than in *Soraria* and *Aria* samples (Table 2). Moreover, certain compounds were only detected in *S. torminalis* and its hybrid cytotypes (Table 2), compared to the members of *Aria* (including all cytotypes) and *Soraria*. The large divergence between *S. torminalis* and *Tormaria* is primarily due to the presence of ‘discriminating’ compounds (apigenin 6,8-di-C-glucoside, luteolin 7-O-glucoside, luteolin 7-O-glucuronide, luteolin hexoside, apigenin 7-O-glucuronide).

The relationships between the parental species and the derived hybrids within the subg. *Tormaria* and *Soraria* are even clearer (Figure 3B,C). *Sorbus torminalis* acts as the maternal parent, as in most hybridogen taxa of the subg. *Tormaria* [4,13,52,53], suggesting a maternal effect in the inheritance of flavonoid compounds. The strong parental differentiation between *S. torminalis* on the one hand and *S. aria* on the other is due to the ‘discriminating’ compounds that are completely absent in the subgenus *Aria* (Table 2). These are derivatives of luteolin: luteolin-7-O-glucoside (10), luteolin-7-O-glucuronide

(11) and luteolin hexoside (20), characteristic compounds for the EtOAc fraction of *S. torminalis* × *S. aria* and the corresponding parental species *S. torminalis*. On the other hand, the derivatives of apigenin: apigenin-7-O-glucuronide (21) and apigenin-6-C-glucoside-8-C-arabinoside (4) are also characteristic compounds only for the EtOAc fraction of *S. torminalis* × *S. aria* and the corresponding parental species *S. torminalis*. The apigenin 6,8-di-C-glucoside (2) is exclusively present in the ethyl acetate fraction of *S. torminalis* × *S. aria*, but not in the parental species *S. torminalis*. In contrast to the previously mentioned observation, kaempferol 3-O-glucoside (16) is exclusively detected in the parental lineage *S. torminalis*, but is completely absent in the individuals of the hybrid *S. torminalis* × *S. aria*. The presence or absence of apigenin and luteolin derivatives allowed a clear distinction between the studied groups *Aria*, *Tormaria* and *Soraria*. These flavonoids, known for their biological activity, can serve as markers for the differentiation of the various *Sorbus* accessions. Within *Tormaria*, a complete absence of methylquercetin acetylhexoside (23) and quercetin pentoside (15) was found. Although the *Tormaria* accessions share most flavonoid compounds with the parental species, they also possess two exclusively novel compounds: (21) methylquercetin hexoside isomer 1 and (1) apigenin 6,8-di-C-glucoside (Table 2). Feulner et al. [53] found that the German *Tormaria* samples contained mixtures of the parental compounds; however, novel compounds were not detected.

The relationships between the *Soraria* samples and their parental species, particularly *S. austriaca* and *S. bosniaca*, were less clear (Figures 3B and 4A,B). The samples of *S. austriaca* were more closely related to *S. aucuparia*, but surprisingly distinct from *S. bosniaca* (Figures 2A, 3B and 4A,B). The flavonoid composition of *S. austriaca* and *S. bosniaca* had only six common compounds (rutin, hyperoside, isoquercitrin, quercetin hexosylpentoside and kaempferol acetylhexoside, Table 2), whereas *S. bosniaca* also contains the three compounds found in *S. torminalis*, *Tormaria* subg. and *S. aria* cytotypes (quercetin deoxyhexosyl hexoside (18), methylquercetin hexoside isomer 1 (21) and methylquercetin hexoside isomer 2 (22), Table 2). In addition, the compound quercetin dihexoside (3) is found exclusively in *S. austriaca* and *S. aucuparia*, whereas it is completely absent in the subg. *Aria* and *S. bosniaca*. While the component quercetin acetylhexoside (15) is uniquely present in both samples of *S. austriaca*, quercetin trideoxyhexoside (5) and methylquercetin acetylhexoside (23) are detected in a single individual of *S. austriaca* (Mt. Umoljani). The absence of these compounds in the second *S. austriaca* sample is intriguing, as most Balkan populations represent a single genetic clone due to apomixis [54]. This finding suggests a possible independent origin of these two *S. austriaca* samples. In addition, a triploid *S. aria* sample (Mt. Umoljani) shares these two compounds with *S. austriaca* (Table 2), suggesting a possible hybridisation between the two species. The most common way to form *Sorbus* polyploids is by crossing a polyploid formation, whereas tetraploid apomict is crossed with a sexual diploid to produce a triploid apomict [5,55–57]. Therefore, such a scenario is likely, as the Umoljani site represents a large sympatric community of *S. aria* cytotypes and *S. austriaca*, which has been well studied in terms of ploidy dynamics and reproductive mode [57].

Similar patterns with respect to the presence or absence of quercetin derivatives are shown for methylquercetin acetylhexoside (23), quercetin deoxyhexosyl hexoside (6) and quercetin hexosylpentoside (13) in *S. austriaca* and *S. bosniaca* (Table 2). Such a variable composition of flavonoids in two closely related species that share at least one parental species may be due to several factors. First, both species originated from hybridisation between subg. *Aria* and *S. aucuparia* and represent the allotetraploid lineages, but their exact origin remains unclear [54,57]. In most taxa of the subg. *Soraria*, *S. aucuparia* is the maternal parent and the pollen donors are various members of the subg. *Aria*, whose identity remains unknown in most cases [4,14,52,55,57]. Therefore, the significantly different flavonoid profiles of these two species are not surprising (Table 2), which is due

to the unknown paternal parent as well as the allopolyploid origin of these taxa. Each polyploid hybridisation is an independent event that can significantly restructure the genome, alter gene expression and induce physiological, phenotypic and biochemical changes [58,59]. Second, the diversity in flavonoid composition could be attributed to the parental influence of the subg. *Aria* members in hybridisation events, but this should be interpreted with caution and requires further research.

The effects of hybridisation on the occurrence of flavonoids differ within the subg. *Soraria* and *Tormaria*. The presence/absence of quercetin derivatives is associated with the subg. *Soraria*, whereas the variation of luteolin, apigenin and kaempferol derivatives is associated with the subg. *Tormaria*. In addition, *S. aria* as the paternal parent has a significant influence on the qualitative variation within the *Soraria* group in both subg., while *S. torminalis* plays a more important role in the subg. *Tormaria*. This phenomenon is probably also related to the biosynthetic pathway of the flavonoids. The pathway starts with p-coumaroyl-CoA and malonyl-CoA, which are condensed by chalcone synthase (CHS) to 2,4,6,4-tetrahydroxy chalcone. Chalcone is then converted to naringenin by chalcone isomerase (CHI) [60]. From this point on, naringenin becomes a precursor for all the different biosynthetic pathways. The different biosynthetic pathways depend on the action of key enzymes such as flavone synthase (FSN) and flavonol synthase (FLS). When FSN is active, naringenin is converted into flavones. When FLS is active, naringenin is hydroxylated to form dihydroflavonols, which are precursors of flavanols [61]. Based on the above results and the absence of flavone derivatives in the *Soraria* samples, it can be concluded that the expression of the FSN enzyme is either repressed or inhibited, in contrast to the *Tormaria* samples. The question of whether the enzyme has been lost in the *Soraria* species or whether they have ever acquired it has not yet been clarified [62].

Certain individuals, both in the subg. *Soraria* and in the subg. *Tormaria*, produced novel compounds compared to the parental individuals. This is considered a rare example of hybridisation and polyploidy influencing the qualitative variation of secondary metabolites. Inheritance of secondary metabolites often conforms to Mendelian principles, where the presence of a secondary metabolite occurs when at least one parent produces it, resulting in complementary chemical patterns. However, deviations from this pattern are due to changes in the biosynthetic pathway [63]. These changes may include the addition or loss of genes or alleles that lead to new biochemical properties [17,63]. Blockages in the metabolic pathway can lead to the accumulation of intermediates, or unique interactions between enzymes and genes from each parent can lead to novel compounds [64]. Mutations in regulatory genes during hybridisation can also alter where or how secondary metabolites are produced, leading to greater biochemical diversity. On the other hand, the quantitative characteristics of secondary metabolites are controlled by multiple genes and influenced by dominant, recessive, additive or epistatic interactions [65]. F1 hybrids generally exhibit intermediate levels of secondary metabolite expression, with additive inheritance being the most common pattern. Additive effects account for 56% of the variation in secondary metabolite expression [17,65]. This variation can result from allelic differences affecting enzymes in the biosynthetic pathway or regulatory genes [17].

### 3.3. No Polyploid Effect on Flavonoid Profiles in *S. aria* Cytotypes

The production of secondary metabolites associated with ploidy is still a poorly understood aspect of plant biology. Polyploidy can lead to the differential regulation of biosynthetic pathways, affecting both the concentrations of biosynthetic products and the presence of certain metabolites. Studies indicate that tetraploids accumulate higher concentrations of phenylpropanoid compounds compared to diploid progenitors [66–68]. However, the concentrations

of individual metabolites in polyploids can sometimes be lower than expected [68]. Our results, based on the Mantel test, generally showed a weak association of flavonoid profiles with the increase in ploidy in the *Sorbus* samples analysed. This pattern has been previously confirmed in other species [69–73]. On the other hand, no correlation was found between the increase in ploidy and the flavonoid profiles in samples of the subg. *Aria*. The *Aria* samples in this study exhibited a wide diversity of flavonoid profiles, regardless of ploidy level and geographical origin (Figures 3C and 4A,B). The highest divergence among the diploid samples is particularly surprising. This pattern suggests that geographically determined flavonoid diversity is due to geographic origin [74]. Triploids and tetraploids showed less divergence (Figures 3C and 4A,B), with the latter being the most homogeneous group within the analysed *Aria* group. The polyploids of the subg. *Aria* originated by hybridisation or polyploidy from diploid *S. aria* s. str. interacting with polyploids [8,75]. Therefore, the autopolyploid *versus* the allopolyploid origin of the *Aria* polyploids is probably also an important factor in shaping the flavonoid diversity within this group. The grouping pattern of *S. aria* tetraploids in this study was found to be the most homogeneous group in terms of flavonoid compounds (Figures 3C and 4A; Table 2), suggesting an autopolyploid origin. It has been observed that the concentration of secondary metabolites increases in autopolyploids, probably due to a gene dosage effect [76,77], but this is not the case in the tetraploid samples.

In allopolyploids, it is assumed that the combination of biosynthetic complements from two progenitor species increases the variation in the resulting production of secondary metabolites [77]. Allopolyploidisation events can also lead to an organism that can produce all enzymes and metabolites of the progenitor species [78]. A high degree of divergence was observed in triploid *S. aria* samples (Figures 3C and 4A,B), suggesting a possible allotriploid origin for at least two of them. This is particularly indicative of triploids of *S. aria* from Umljani and Bijela gora, which intermix with *S. austriaca* (Figure 4A,B). Triploids of *S. aria* are most likely products of pollen exchange from apomictic tetraploids to sexual diploids, a pathway of triploid formation via the tetraploid bridge [5,9,57]. In the case of the two triploid *S. aria*, the potential pollen donor may be the tetraploid *S. austriaca* or *S. aria* [59]. The triploid *S. aria* from Mt. Igman is in the clade with the diploid and tetraploid *S. aria* samples from Mt. Igman (Figure 4A), indicating a probable autotriploid origin. The discovery of novel compounds in series of polyploid *S. aria* (methylquercetin acethylhexoside (**23**) in the diploid; hydroxyquercetin deoxyhexosyl hexoside (**1**) in the triploid; kaempferol 3-*O*-glucoside (**16**) and methylquercetin hexoside isomer 2 (**19**) in the tetraploid) suggests an allopolyploid origin of the triploids and tetraploids [69]. The comparison of the flavonoid profiles of *S. aria* from Mt. Igman shows that eight flavonoid compounds occur in all three ploidy levels. Of these, only one, quercetin-3-*O*-glucoside (**9**), shows an increase in the tetraploid sample, while other flavonoids do not follow the pattern of correlation between ploidy level and flavonoid content. In addition, a novel compound, quercetin 3-*O*-galactoside (**8**), was identified in the tetraploids. Of these, only one compound, quercetin 3-*O*-rutinoside (**7**), shows an increase in the tetraploid sample of *S. aria* from Bijela gora, while other flavonoids do not follow the pattern of correlation between ploidy level and flavonoid content. In addition, a novel compound, methylquercetin acethylhexoside (**23**), was found in the diploid *S. aria*, hydroxyquercetin deoxyhexosyl hexoside (**1**) in the triploid *S. aria* and kaempferol 3-*O*-glucoside (**16**), methylquercetin hexoside isomer 2 (**19**) in the tetraploid *S. aria* were identified.

## 4. Materials and Methods

### 4.1. Plant Material

Leaf material of *Sorbus* accessions (species and cytotypes) from 10 locations in Bosnia and Herzegovina was collected in July 2022 and prepared for phytochemical and cytometric



analyses (Table 3). The identity of the studied taxa was confirmed by Faruk Bogunić and Alma Hajrudinović (Faculty of Forestry, University of Sarajevo, Bosnia and Herzegovina). The sampling design included the following *Sorbus* accessions: parental species (di-, tri- and tetraploids of *S. aria*; diploid *S. torminalis* and *S. aucuparia*) and their di-, tri- and tetraploid hybrid derivatives from *S. torminalis* × *S. aria* crosses (*S. subg. Tormaria*) and tetraploid *S. austriaca* and *S. bosniaca* originated from *S. aria* × *S. aucuparia* crosses (*S. subg. Soraria*) (Table 3). The nomenclature system for the classification of subgenera followed Májovsky and Bernátová [79]. For this study, polyploid *S. aria* and *S. torminalis* × *S. aria* individuals were treated as cytotypes, as these entities have not yet been circumscribed. Detailed information of the geographic origin, genome size (GS) and ploidy level of the *Sorbus* accessions analysed is provided in Table 3. Fresh leaves were used for the cytometric analyses, while the remaining collected raw material was dried at a temperature of 20–25 °C and stored under dark, dry conditions.

**Table 3.** Geographic origin, genome size and ploidy level of studied *Sorbus* accessions.

| Taxon                              | Subgenus                                            | Genome Size (2C pg) | DNA Ploidy Level | Location                 | Voucher Number | North     | East      | Altitude (m) |
|------------------------------------|-----------------------------------------------------|---------------------|------------------|--------------------------|----------------|-----------|-----------|--------------|
| <i>S. aria</i>                     | <i>Aria</i>                                         | 1.47                | 2x               | Grkarica, Mt. Igman      | SARA (54276)   | 43.739167 | 18.291389 | 1350         |
|                                    |                                                     | 1.41                | 2x               | Mt. Bijela gora          | SARA (54280)   | 42.677778 | 18.475833 | 730          |
|                                    |                                                     | 1.41                | 2x               | Gradac, Posušje          | SARA (54278)   | 42.425    | 17.3925   | 720          |
|                                    |                                                     | 2.12                | 3x               | Grkarica, Mt. Igman      | SARA (51415)   | 43.739167 | 18.291389 | 1350         |
|                                    |                                                     | 2.11                | 3x               | Umoljani, Mt. Bjelašnica | SARA (51412)   | 43.664167 | 18.226111 | 1300         |
|                                    |                                                     | 2.11                | 3x               | Mt. Bijela gora          | SARA (54277)   | 42.677778 | 18.475833 | 730          |
|                                    |                                                     | 2.82                | 4x               | Grkarica, Mt. Igman      | SARA (51416)   | 43.739167 | 18.291389 | 1350         |
|                                    |                                                     | 2.80                | 4x               | Umoljani, Mt. Bjelašnica | SARA (51413)   | 43.664167 | 18.226111 | 1300         |
|                                    |                                                     | 2.78                | 4x               | Mt. Bijela gora          | SARA (54279)   | 42.677778 | 18.475833 | 730          |
| <i>S. aucuparia</i>                | <i>Sorbus</i>                                       | 1.43                | 2x               | Veliko polje, Mt. Igman  | SARA (51417)   | 43.745278 | 18.275    | 1210         |
| <i>S. austriaca</i>                | <i>Soraria</i> ( <i>Sorbus</i> × <i>Aria</i> )      | 2.81                | 4x               | Grkarica, Mt. Igman      | SARA (51418)   | 43.739167 | 18.291389 | 1350         |
|                                    |                                                     | 2.77                | 4x               | Umoljani, Mt. Bjelašnica | SARA (51414)   | 43.664167 | 18.226111 | 1300         |
| <i>S. bosniaca</i>                 |                                                     | 2.80                | 4x               | Mt. Krug planina         | WU (080424)    | 43.842222 | 17.199722 | 1300         |
| <i>S. torminalis</i> × <i>aria</i> | <i>Tormaria</i> ( <i>Torminalis</i> × <i>Aria</i> ) | 1.40                | 2x               | Pratača, Mt. Igman       | SARA (54281)   | 43.763611 | 18.19138  | 915          |
|                                    |                                                     | 2.18                | 3x               | Koznik                   | SARA (54282)   | 43.7125   | 17.9675   | 900          |
|                                    |                                                     | 2.74                | 4x               | Crne lokve, Posušje      | SARA (54283)   | 43.4425   | 17.464444 | 700          |
| <i>S. torminalis</i>               | <i>Torminaria</i>                                   | 1.44                | 2x               | Tihovići                 | SARA (54284)   | 43.923889 | 18.377778 | 910          |

#### 4.2. Determination of Ploidy Level

Ploidy determination was performed according to the protocol of Hajrudinović et al. [59] using flow cytometry. In brief, fresh leaves of selected individuals from 10 populations (Table 3) were co-chopped with fresh leaves of the internal standard, *Medicago truncatula* Gaertn. cv. R108-1 (0.98 pg, Marie and Brown [80], in 600 mL of cold Gif nuclear buffer [81]. The suspension was filtered through a 50 µm nylon sieve (CellTrics, Partec), and RNase (Roche) was added to 25 U mL<sup>-1</sup>. The cell nuclei were stained with propidium iodide (Sigma-Aldrich) at a final concentration of 50 mg mL<sup>-1</sup> and incubated on ice for 5 to 10 min before analysis. The fluorescence of approximately 3000 cell nuclei was recorded for each sample using either a Partec CyFlow SL3 (Partec, Münster, Germany) 532 nm laser cytometer or a CyFlow Ploidy Analyser (Sysmex Europe SE) 532 nm laser. The 2C DNA values were determined and the DNA ploidy level [82] were derived by comparing these values with those of individuals with known chromosome number [83].

#### 4.3. Sample Preparation for LC-MS Analysis

Dried leaves of *Sorbus* accessions were pulverized and extracted with methanol, which served as a suitable extraction solvent at 20 °C, with two cycles of 20 min each using ultrasound. The drug-to-solvent ratio was 1:40. The methanolic extracts were centrifuged at 4000 rpm for 20 min at 4 °C. The prepared methanolic extract was evaporated to dryness, reconstituted in water at a 1:100 ratio, and then subjected to successive liquid–liquid extractions with solvents of increasing polarity: (a) hexane, (b) diethyl ether and (c) ethyl acetate. The ethyl acetate fractions (EtOAc) were collected, evaporated to dryness, and dissolved in 1 mL of HPLC-grade methanol prior to analysis [84].

#### 4.4. LC-MS Analysis

LC-MS analysis of EtOAc fractions of *Sorbus* leaves obtained from MeOH extracts was performed on an Agilent LC/MS system 1260/6130 (Agilent Technologies, Waldbronn, Germany) with diode array detector (DAD) and single quadrupole API-ESI MSD. A Zorbax SB-Aq column (150 × 3.0 mm; 3.5 µm particle size, Agilent Technologies), and a binary mobile phase (A—formic acid, 0.1%, *v/v*; B—acetonitrile) were used for chromatographic separation, and the gradient program, column temperature, flow rate, injection volume and DAD and MSD parameters were determined based on previous work [85]. The obtained dry fraction/extracts were dissolved in MeOH (final working concentrations were 5 mg/mL) and filtered through a cellulose membrane filter (0.45). The recorded raw data were processed using ChemStation (Rev. B.04.03-SP1) software. The external standard method was used for quantitative analysis and the levels of the detected compounds were calculated based on the peak areas obtained by DAD (at 350 nm) using calibration curves obtained for ten commercial standards (regression equations, correlation coefficients (*r*<sup>2</sup>), linear ranges, LODs and LOQs in µg/mL are given in the Table S1. Following the guidelines of the International Conference on Harmonization (ICH, 2005), the LODs and LOQs were determined using the signal-to-noise ratio as described in the European Pharmacopoeia (Ph. Eur. 11.0) [73,86]. The analysis was performed in triplicate. The amounts of the detected compounds were calculated as gram equivalents of the standards used per 100 g of dry fraction (dw) due to their high structural similarity.

#### 4.5. Chemicals and Reagents

LC-MS-grade formic acid and acetonitrile, as well as standards (of HPLC purity): Rutin, Isoquercitrin, Quercitrin, Luteolin 7-*O*-glucoside, Luteolin 7-*O*-glucuronide, Astragalgin, Apigenin 6,8-di-*C*-glucoside (Vicenin), Apigenin-6-*C*-glucoside-8-*C*-arabinoside

(Schafotoside) and propidium iodide (Sigma-Aldrich, Merck Group, Merck KGaA, Darmstadt, Germany), Quercetin 3-O-galactoside (Carl Roth, Karlsruhe, Germany), Apigenin 7-O-glucuronide from HWI Analytik (Ruelzheim, Germany).

#### 4.6. Data Analysis

Descriptive statistics (mean and standard deviation for each of the analysed compounds at the individual level), univariate statistics (one-way ANOVA followed by a Tukey HSD test) and multivariate techniques, including Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA), non-metric multidimensional scaling (nMDS) and Cluster Analysis (CA) were applied to assess the variation of flavonoid compounds. Triplicate measurements were averaged for each individual to create a data matrix. To reduce the magnitude of differences in the original values (the amounts of compounds expressed as relative percentages) and to avoid making assumptions about the data distribution, the data were coded (using categorical scale) following Milutinović et al. [87]. The aim of the PCA was to reveal a general pattern of variations and relationships between all individuals. To hypothetically reduce the influence of environmental factors on the production of flavonoid compounds in studied samples, we generated a binary data matrix for the presence/absence of compounds. We then performed separate PCoAs for all individuals, for the *Tormaria* and *Soraria* groups, including their putative parental species and their hybrid members (species and cytotypes), and for the *Aria* group. The PCoAs based on Jaccard distances were used to visualise the relationships between individuals. The cluster analysis by the unweighted pair-group method using arithmetic averages (UPGMAs) using Jaccard distances was performed to verify the affinities between samples. Finally, NMDS using the Bray–Curtis pairwise distance matrix was used to order the studied samples. The Mantel test [88] was used to check whether the quantitative composition of the flavonoid components is affected by the level of ploidy for all accessions, particularly for the *Aria* subgenus. The Bray–Curtis distances for raw phytochemical data and ploidy data were used for calculation. All analyses were performed using PAST 3.14 [89].

## 5. Conclusions

The results of HPLC-MS and flow cytometry proved valuable for the analysis of ploidy-dependent plant systems and the identification of flavonoid profiles in a panel of *Sorbus* accessions, including three diploid parental species and polyploid derivatives of the subg. *Tormaria*, *Soraria* and *Aria* from the region of Bosnia and Herzegovina. Of the 23 flavonoids identified, 14 were newly reported, improving the understanding of the phytochemical profiles of *Sorbus* species. We have shown that certain fractions of flavonoids are reliable indicators of hybridisation in certain *Sorbus* groups (*Tormaria* and *Soraria*). Correlation with ploidy levels reveals a non-statistically significant relationship with flavonoid profiles in a range of *S. aria* polyploids. Flavonoid profiling highlights the *Tormaria* group as target accessions rich in bioactive compounds for further biological studies.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/plants14010119/s1>, Table S1. Calibration curves regression equations, correlation coefficients ( $r^2$ ), linear ranges, LODs and LOQs of reference compounds, Table S2. Principal components (PC) revealed by Principal Component Analysis (PCA) for the studied *Sorbus* accessions.

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B. and F.B.; writing—original draft preparation, E.K., F.B., V.M., I.G. and A.H.-B.; writing—review and editing, E.K., F.B., V.M., I.G. and A.H.-B.; visualisation, A.H.-B., V.M. and E.K.; supervision H.N. and F.B. All authors have read and agreed to the published version of the manuscript.

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## Article

# Chemodiversity and Antinociceptive Activity of *Amorpha fruticosa* L. Essential Oil

Miljana R. Đorđević Zlatković <sup>1</sup>, Nikola M. Stojanović <sup>2</sup>, Dragan B. Zlatković <sup>1</sup>, Pavle J. Randjelović <sup>2</sup> and Niko S. Radulović <sup>1,\*</sup>

<sup>1</sup> Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, 18000 Niš, Serbia; miljana.djordjevic@pmf.edu.rs (M.R.Đ.Z.); dragan.zlatkovic@gmail.com (D.B.Z.)

<sup>2</sup> Department of Physiology, Faculty of Medicine, University of Niš, 18000 Niš, Serbia; nikola.st90@yahoo.com (N.M.S.); pavleus@gmail.com (P.J.R.)

\* Correspondence: nikoradulovic@yahoo.com

**Abstract:** An essential oil dominated by germacrene D (19.3% by GC) was isolated from the fresh fruit of *Amorpha fruticosa* L. (Fabaceae). Agglomerative clustering and *k*-means clustering were employed to compare the composition of the oil with the existing literature data, suggesting that the *A. fruticosa* used in this study represents a new chemotype. The essential oil was evaluated for its antinociceptive activity using the acetic acid-induced writhing test in rats at doses of 400, 200, and 100 mg/kg. All tested doses reduced the number of writhes induced by the intraperitoneal injection of acetic acid. The 400 mg/kg dose of the oil demonstrated a 54.4% inhibition, which was statistically different from the positive control, aspirin, which showed 90.2% inhibition at a dose of 200 mg/kg. Since the injection of acetic acid produces the release of prostaglandins, such as PGE2 $\alpha$  and PGF2 $\alpha$ , as well as sympathetic nervous system mediators in peritoneal fluids, the results suggest that the inhibition of prostaglandin release might represent one of the possible mechanisms of action exerted by the oil.

**Keywords:** *Amorpha fruticosa*; essential oil; antinociceptive activity; clustering analysis

## 1. Introduction

*Amorpha fruticosa* L. (Fabaceae) is a perennial deciduous shrub native to North America [1], known as (desert) false indigo-bush, which is related to the application of *A. fruticosa* as a dye source [2,3]. It was introduced to Europe as an ornamental plant (in the early 1700s); today, it grows naturalized outside of its native range, mostly near river banks, and is planted to stabilize soil for erosion control [1]. *Amorpha fruticosa* is one of the most dangerous invasive species in many European countries. Therefore, its exploitation could offer an affordable resource of potentially valuable chemicals. For example, its fruits are used in the perfumery and pharmaceutical industries in Bulgaria [3,4].

Traditionally, false indigo-bush has been used in Chinese medicine to treat hypertension, hematomas, contusions, burns, eczema, stomach pain, and neuralgia, which suggests an application of its secondary metabolites for medicinal purposes [5–7]. For a comprehensive overview of the (ethno)medicinal and non-medicinal uses of *A. fruticosa*, we refer readers to the review by Grabić et al. [7].

Previous phytochemical studies of this species resulted in the identification of family-marker classes of compounds such as isoflavonoids and their derivatives rotenoids [8–11], flavanones [12–14], and isoflavones [15], and prenylated stilbenoids [16]. Some metabolites of this plant are rare or unique to the false indigo-bush. The composition of the essential oils from *A. fruticosa* fruits and flowers has been investigated on several occasions. Depending on the location of plant material collection and the specific plant part subjected to analysis, the major components of the oils differed markedly. The essential oil from the fruits contained  $\gamma$ -muurolene and *ar*-curcumene, or  $\delta$ -cadinenes,  $\alpha$ -zingiberene and  $\alpha$ -eudesmol, in samples from Bulgaria and Romania, respectively [5,17].  $\alpha$ -Eudesmol,  $\beta$ -eudesmol,

$\delta$ -cadinene and (*E*)-nerolidol or  $\alpha$ -pinene and myrcene were the most abundant in the flower and ripe fruit oils from Poland; the leaf oil contained  $\alpha$ -eudesmol, (*E*)- $\beta$ -ocimene, and  $\alpha$ -pinene [18]. All these studies reported high yields of isolated oil (up to 1.8% for dried fruit, [5]).  $\gamma$ -Amorphene, a sesquiterpenoid isolated from this species from the first time, has been present only in small amounts in samples collected in Ukraine [19].

Several of the identified compounds from *A. fruticosa* have demonstrated significant antimicrobial activity [20], as well as NF  $\kappa$ B- and neuraminidase, proliferative [2] and TNF- $\alpha$ -inhibitory [13] effects. Extracts of *A. fruticosa* have also been shown to possess antidiabetic activity in diet-induced obese and db/db mice via the activation of the selective peroxisome PPAR $\gamma$  [21]. This activity was attributed to the amorfrutins present in this plant species. The exceptional properties of these natural products could potentially lead to their future biomedical application, either as lead compounds in drug discovery or as reference compounds in pharmacological research [16]. In addition to these activities, Wu and coworkers [16] found a large number of rotenoid glycosides with potent cytotoxic action against MCF-7 and HCT-116 cells.

Considering the abundance of *A. fruticosa* in many regions, its high content of volatile compounds (and thus the potential to obtain large quantities of fruit essential oil from foraged plant material), and the fact that the pharmacological properties of *A. fruticosa* essential oil have been investigated in only two studies (antimicrobial [5] and cytotoxic [22]), the aims of this work were:

- (1) Detailed chemical analysis of *A. fruticosa* fruit essential oil (from Serbia) by GC and GC-MS (gas chromatography–mass spectrometry).
- (2) Statistical chemotypification of the *A. fruticosa* essential oil using clustering analysis, based on current and previously published data.
- (3) An investigation of the antinociceptive activity of the oil in the acetic acid-induced writhing test. Despite the extensive data on the pharmacology of *A. fruticosa*, no study to date has addressed the analgesic activity of this species.

## 2. Results and Discussion

### 2.1. Chemical Composition of *A. fruticosa* Essential Oil

The list of identified volatile constituents of *A. fruticosa* fruits collected in Niš, Serbia, is compiled in Table 1. GC and GC-MS analyses enabled the identification and quantification of 105 components, comprising 92.9% of the total GC peak area detected. The sesquiterpenoid fraction was the most prominent, accounting for 83.8% of the essential oil, unevenly distributed between hydrocarbons (69.8%) and oxygenated derivatives (14.0%), followed by monoterpenoids (8.9%). The main constituents of the essential oil were germacrene D (19.3%),  $\alpha$ -zingiberene (9.9%),  $\delta$ -amorphene (7.6%),  $\gamma$ -muurolene (6.5%), (*E*)-caryophyllene (5.4%), and  $\alpha$ -eudesmol (4.5%). These results align with most existing studies on fruit essential oil [5,16,17], which also reported sesquiterpene hydrocarbons as the main constituents of *A. fruticosa* fruit essential oil. The only outlier was the study by Lis and Góra [18], which identified  $\alpha$ -pinene (ca. 20%) as the main constituent in the monoterpene-dominated essential oil from Polish *A. fruticosa*. The observed yield (1.3%, *w/w*) was significantly higher than that previously reported for fresh fruits by Lis and Góra (0.45%, [18]).

One major difference we immediately observed was that the major component of our oil, germacrene D, was present in levels  $\leq 10\%$  [17] or entirely absent in other studies of *A. fruticosa* fruit oil (see *amorpha\_fruticosa\_dataset.csv* in the Supplementary Materials). The implications of this discrepancy are further discussed in Section 2.2, where we provided statistical evidence strongly suggesting that the *A. fruticosa* examined in this study represents a previously unreported chemotype.

**Table 1.** Chemical composition of the essential oil isolated from fruits of *A. fruticosa*.

| No. <sup>a</sup> | Compound                             | RI <sup>b</sup> | Content [%] <sup>c</sup> | Compound Class <sup>d</sup> | Identification Method <sup>e</sup> |
|------------------|--------------------------------------|-----------------|--------------------------|-----------------------------|------------------------------------|
| 1                | 1-Pentanol                           | 764             | tr                       | O                           | MS, RI, Col                        |
| 2                | Hexanal                              | 801             | 0.2                      | O                           | MS, RI, Col                        |
| 3                | 1-Hexanol                            | 863             | tr                       | O                           | MS, RI, Col                        |
| 4                | Heptanal                             | 901             | tr                       | O                           | MS, RI, Col                        |
| 5                | $\alpha$ -Thujene                    | 921             | tr                       | MT                          | MS, RI                             |
| 6                | $\alpha$ -Pinene                     | 930             | 4.2                      | MT                          | MS, RI, Col                        |
| 7                | Camphene                             | 948             | tr                       | MT                          | MS, RI, Col                        |
| 8                | (E)-2-Heptenal                       | 948             | tr                       | O                           | MS, RI                             |
| 9                | Benzaldehyde                         | 959             | tr                       | O                           | MS, RI, Col                        |
| 10               | Sabinene                             | 969             | tr                       | MT                          | MS, RI                             |
| 11               | 1-Octen-3-ol                         | 970             | tr                       | O                           | MS, RI                             |
| 12               | $\beta$ -Pinene                      | 977             | 0.7                      | MT                          | MS, RI, Col                        |
| 13               | Myrcene                              | 982             | 1.0                      | MT                          | MS, RI                             |
| 14               | 2-Pentylfuran                        | 983             | tr                       | O                           | MS, RI                             |
| 15               | $\alpha$ -Phellandrene               | 1005            | tr                       | MT                          | MS, RI                             |
| 16               | $\alpha$ -Terpinene                  | 1015            | tr                       | MT                          | MS, RI, Col                        |
| 17               | <i>o</i> -Cymene                     | 1021            | 0.1                      | MT                          | MS, RI, Col                        |
| 18               | Limonene                             | 1026            | 0.3                      | MT                          | MS, RI, Col                        |
| 19               | (Z)- $\beta$ -Ocimene                | 1028            | 1.4                      | MT                          | MS, RI, Col                        |
| 20               | 1,8-Cineole                          | 1030            | tr                       | MT *                        | MS, RI, Col                        |
| 21               | (E)- $\beta$ -Ocimene                | 1039            | 0.2                      | MT                          | MS, RI, Col                        |
| 22               | Phenylacetaldehyde                   | 1039            | tr                       | O                           | MS, RI, Col                        |
| 23               | $\gamma$ -Terpinene                  | 1053            | 0.1                      | MT                          | MS, RI, Col                        |
| 24               | <i>cis</i> -Sabinene hydrate         | 1066            | tr                       | MT                          | MS, RI                             |
| 25               | Terpinolene                          | 1083            | tr                       | MT                          | MS, RI, Col                        |
| 26               | Linalool                             | 1094            | 0.3                      | MT *                        | MS, RI, Col                        |
| 27               | <i>trans</i> -Sabinene hydrate       | 1100            | 0.1                      | MT                          | MS, RI, Col                        |
| 28               | Nonanal                              | 1100            | tr                       | O                           | MS, RI, Col                        |
| 29               | Isopentyl isovalerate                | 1102            | tr                       | O                           | MS, RI, Col                        |
| 30               | <i>trans</i> -Pinocarveol            | 1139            | tr                       | MT *                        | MS, RI                             |
| 31               | <i>trans</i> -Verbenol               | 1143            | tr                       | MT *                        | MS, RI                             |
| 32               | Isopulegol                           | 1146            | tr                       | MT *                        | MS, RI                             |
| 33               | <i>iso</i> -Isopulegol               | 1156            | tr                       | MT *                        | MS, RI                             |
| 34               | (E)- 2-Nonen-1-ol                    | 1164            | tr                       | O                           | MS, RI                             |
| 35               | Terpinen-4-ol                        | 1175            | 0.2                      | MT *                        | MS, RI, Col                        |
| 36               | $\alpha$ -Terpineol                  | 1192            | tr                       | MT *                        | MS, RI, Col                        |
| 37               | Citronellol                          | 1220            | 0.3                      | MT *                        | MS, RI, Col                        |
| 38               | (2E,4E)-2,4-Decadienal               | 1314            | tr                       | O                           | MS, RI                             |
| 39               | $\alpha$ -Cubebene                   | 1343            | 0.7                      | ST                          | MS, RI                             |
| 40               | $\alpha$ -Ylangene                   | 1366            | 0.7                      | ST                          | MS, RI                             |
| 41               | $\alpha$ -Copaene                    | 1372            | 2.6                      | ST                          | MS, RI                             |
| 42               | $\beta$ -Bourbonene                  | 1381            | tr                       | ST                          | MS, RI                             |
| 43               | 7- <i>epi</i> -Sesquithujene         | 1382            | 1.2                      | ST                          | MS, RI                             |
| 44               | $\beta$ -Cubebene                    | 1384            | tr                       | ST                          | MS, RI                             |
| 45               | $\beta$ -Elemene                     | 1385            | 1.1                      | ST                          | MS, RI                             |
| 46               | Sesquithujene                        | 1396            | 0.1                      | ST                          | MS, RI                             |
| 47               | $\alpha$ -Gurjunene                  | 1404            | 0.5                      | ST                          | MS, RI                             |
| 48               | <i>cis</i> - $\alpha$ -Bergamotene   | 1408            | tr                       | ST                          | MS, RI                             |
| 49               | $\beta$ -Ylangene                    | 1415            | tr                       | ST                          | MS, RI                             |
| 50               | (E)-Caryophyllene                    | 1417            | 5.4                      | ST                          | MS, RI, Col                        |
| 51               | $\beta$ -Copaene                     | 1427            | 1.4                      | ST                          | MS, RI                             |
| 52               | <i>trans</i> - $\alpha$ -Bergamotene | 1428            | tr                       | ST                          | MS, RI                             |
| 53               | Aromadendrene                        | 1435            | 0.9                      | ST                          | MS, RI                             |
| 54               | Cadina-3,5-diene                     | 1445            | tr                       | ST                          | MS, RI                             |
| 55               | (E)- $\beta$ -Farnesene              | 1446            | 0.5                      | ST                          | MS, RI                             |
| 56               | $\alpha$ -Humulene                   | 1452            | 1.7                      | ST                          | MS, RI, Col                        |
| 57               | <i>cis</i> -Cadina-1(6),4-diene      | 1456            | tr                       | ST                          | MS, RI                             |
| 58               | <i>cis</i> -Muurola-4(14),5-diene    | 1458            | 0.3                      | ST                          | MS, RI                             |
| 59               | <i>trans</i> -Cadina-1(6),4-diene    | 1468            | 0.5                      | ST                          | MS, RI                             |
| 60               | $\gamma$ -Muurolene                  | 1471            | 6.5                      | ST                          | MS, RI                             |
| 61               | <i>ar</i> -Curcumene                 | 1476            | tr                       | ST                          | MS, RI                             |
| 62               | Germacrene D                         | 1479            | 19.3                     | ST                          | MS, RI                             |
| 63               | (Z,E)- $\alpha$ -Farnesene           | 1484            | tr                       | ST                          | MS, RI                             |
| 64               | $\beta$ -Selinene                    | 1486            | 1.3                      | ST                          | MS, RI                             |
| 65               | $\alpha$ -Zingiberene                | 1489            | 9.9                      | ST                          | MS, RI                             |
| 66               | $\alpha$ -Muurolene                  | 1494            | tr                       | ST                          | MS, RI                             |

Table 1. Cont.

| No. <sup>a</sup>               | Compound                                                | RI <sup>b</sup> | Content [%] <sup>c</sup> | Compound Class <sup>d</sup> | Identification Method <sup>e</sup> |
|--------------------------------|---------------------------------------------------------|-----------------|--------------------------|-----------------------------|------------------------------------|
| 67                             | ( <i>E,E</i> )- $\alpha$ -Farnesene                     | 1497            | tr                       | ST                          | MS, RI                             |
| 68                             | $\beta$ -Bisabolene                                     | 1502            | tr                       | ST                          | MS, RI                             |
| 69                             | $\gamma$ -Cadinene                                      | 1509            | 4.2                      | ST                          | MS, RI                             |
| 70                             | Cubebol                                                 | 1511            | tr                       | ST *                        | MS, RI                             |
| 71                             | $\delta$ -Amorphene                                     | 1514            | 7.6                      | ST                          | MS, RI                             |
| 72                             | <i>trans</i> -Calamenene                                | 1516            | tr                       | ST                          | MS, RI                             |
| 73                             | $\beta$ -Sesquiphellandrene                             | 1518            | 2.3                      | ST                          | MS, RI                             |
| 74                             | ( <i>E</i> )- $\gamma$ -Bisabolene                      | 1521            | tr                       | ST                          | MS, RI                             |
| 75                             | 10- <i>epi</i> -Cubebol                                 | 1526            | 0.1                      | ST *                        | MS, RI                             |
| 76                             | <i>trans</i> -Cadina-1,4-diene                          | 1528            | 0.6                      | ST                          | MS, RI                             |
| 77                             | $\alpha$ -Cadinene                                      | 1532            | 0.5                      | ST                          | MS, RI                             |
| 78                             | $\alpha$ -Calacorene                                    | 1537            | tr                       | ST                          | MS, RI                             |
| 79                             | <i>cis</i> -Sesquisabinene hydrate                      | 1537            | 0.7                      | ST *                        | MS, RI                             |
| 80                             | Elemol                                                  | 1543            | 0.9                      | ST *                        | MS, RI                             |
| 81                             | ( <i>E</i> )-Nerolidol                                  | 1553            | 0.3                      | ST *                        | MS, RI                             |
| 82                             | $\beta$ -Calacorene                                     | 1557            | tr                       | ST                          | MS, RI                             |
| 83                             | 1 $\alpha$ ,10 $\alpha$ -Epoxyamorph-4-ene              | 1565            | tr                       | ST *                        | MS, RI                             |
| 84                             | Germacrene D-4-ol                                       | 1572            | tr                       | ST *                        | MS, RI                             |
| 85                             | Spathulenol                                             | 1573            | 2.0                      | ST *                        | MS, RI                             |
| 86                             | Caryophyllene oxide                                     | 1579            | 0.5                      | ST *                        | MS, RI, CoI                        |
| 87                             | Salvial-4(14)-en-1-one                                  | 1588            | tr                       | ST *                        | MS, RI                             |
| 88                             | Globulol                                                | 1591            | 0.6                      | ST *                        | MS, RI                             |
| 89                             | Ledol                                                   | 1601            | tr                       | ST *                        | MS, RI                             |
| 90                             | Rosifolol                                               | 1603            | tr                       | ST *                        | MS, RI                             |
| 91                             | Humulene epoxide II                                     | 1606            | tr                       | ST *                        | MS, RI                             |
| 92                             | Zingiberenol                                            | 1608            | 0.3                      | ST *                        | MS, RI                             |
| 93                             | 1,10-di- <i>epi</i> -Cubenol                            | 1611            | tr                       | ST *                        | MS, RI                             |
| 94                             | Muurola-4,10(14)-dien-1 $\beta$ -ol                     | 1621            | tr                       | ST *                        | MS, RI                             |
| 95                             | 1- <i>epi</i> -Cubenol                                  | 1623            | 0.3                      | ST *                        | MS, RI                             |
| 96                             | Eremoligenol                                            | 1626            | tr                       | ST *                        | MS, RI                             |
| 97                             | $\gamma$ -Eudesmol                                      | 1627            | 1.3                      | ST *                        | MS, RI                             |
| 98                             | <i>epi</i> - $\alpha$ -Cadinol (syn. $\tau$ -cadinol)   | 1637            | 1.2                      | ST *                        | MS, RI                             |
| 99                             | <i>epi</i> - $\alpha$ -Muurolol (syn. $\tau$ -muurolol) | 1639            | tr                       | ST *                        | MS, RI                             |
| 100                            | $\alpha$ -Muurolol (syn. torreyol)                      | 1642            | 0.2                      | ST *                        | MS, RI                             |
| 101                            | $\alpha$ -Eudesmol                                      | 1651            | 4.5                      | ST *                        | MS, RI                             |
| 102                            | <i>epi</i> - $\beta$ -Bisabolol                         | 1665            | 0.2                      | ST *                        | MS, RI                             |
| 103                            | Cadalene                                                | 1667            | tr                       | ST                          | MS, RI                             |
| 104                            | $\alpha$ -Bisabolol                                     | 1679            | 0.9                      | ST *                        | MS, RI, CoI                        |
| 105                            | Amorpha-4,9-dien-2-ol                                   | 1685            | tr                       | ST *                        | MS, RI                             |
| Total identified [%]           |                                                         |                 | 92.9                     |                             |                                    |
| MT—Monoterpenes                |                                                         |                 | 8.1                      |                             |                                    |
| MT *—Oxygenated monoterpenes   |                                                         |                 | 0.8                      |                             |                                    |
| ST—Sesquiterpenes              |                                                         |                 | 69.8                     |                             |                                    |
| ST *—Oxygenated sesquiterpenes |                                                         |                 | 14.0                     |                             |                                    |
| O—Other                        |                                                         |                 | 0.2                      |                             |                                    |

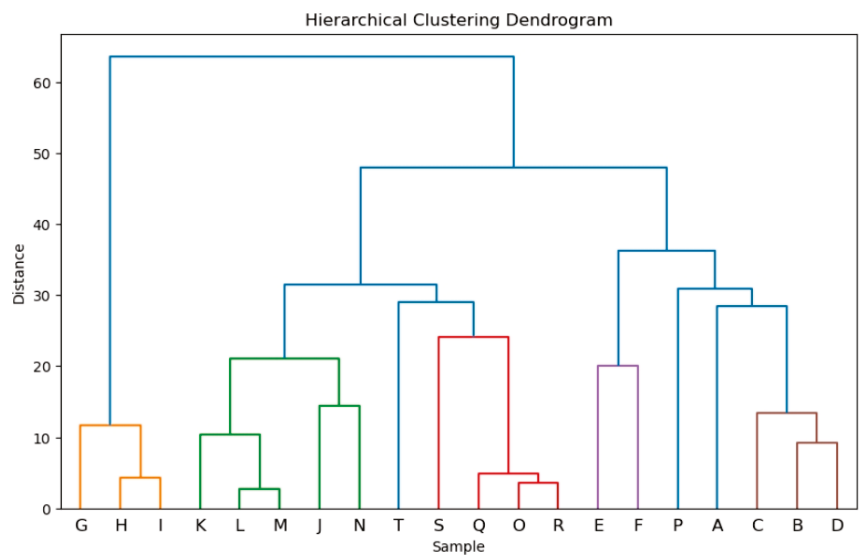
<sup>a</sup> Compounds listed in the order of elution from a DB-5MS column. <sup>b</sup> Linear retention indices (RIs) determined experimentally on the DB-5MS column relative to a series of C<sub>7</sub>–C<sub>17</sub> *n*-alkanes. <sup>c</sup> Values are the means of three individual analyses; tr, trace amounts (<0.05%). <sup>d</sup> The abbreviations of the compound classes are given at the end of the table. <sup>e</sup> Compound identification: RIs, retention indices matching with literature data; MS, mass spectra matching; CoI, coinjection with a pure reference compound.

## 2.2. Clustering Analysis

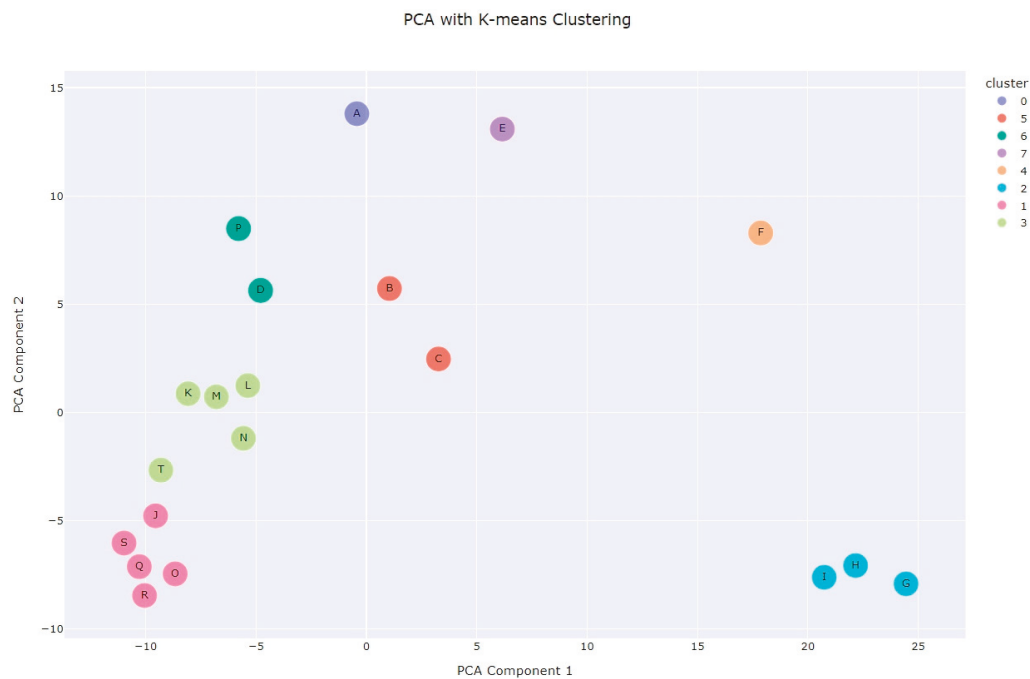
A statistical comparison of *A. fruticosa* essential oil from our study with composition data reported by other authors was performed using *k*-means clustering (following principal component analysis) and agglomerative clustering with Euclidean distances. The results of the analyses are shown in Figures 1 and 2 (the origin of each sample is listed in Table 2). Both methods yielded similar results (see amorpha.html in SI for complete cluster assignments). As expected, samples from the same studies that employed plant material of the same origin mostly grouped into one cluster. This is clearly visualized in the hierarchical clustering dendrogram (Figure 1). Samples I, H, and G (fruit essential oil from Lis and Góra, 2001 [18]; fruit differing in ripeness and freshness) are quite separated from any other sample. Samples J, K, L, M, and N from Stoyanova et al. [17] (different storage times of the



fruit) also formed a distinct cluster. Samples B, C, and D from Ivănescu et al. [5] were all collected in Iași, Romania, and differed only in their collection dates. These observations were expected, as the differences in the handling of the plant materials were not drastic and did not significantly alter the composition of the oil.



**Figure 1.** Hierarchical clustering dendrogram of *A. fruticosa* essential oil samples based on their chemical composition. The color threshold was set at 25. Different colors correspond to different clusters. For details on the sample labels, see Table 2.



**Figure 2.** Principal component analysis (PCA) scatter plot of *A. fruticosa* essential oil samples with *k*-means clustering. The plot shows the distribution of samples along the first two principal components, colored by their respective clusters. For details on the sample labels, see Table 2.

**Table 2.** Labels and origin of *A. fruticosa* essential oil samples used in the clustering analysis.

| Label | Reference                     | Description                                                      |
|-------|-------------------------------|------------------------------------------------------------------|
| A     | Essential oil from this study | fresh ripe fruits/hydrodistillation Clevenger apparatus          |
| B     | Ivănescu et al., 2014 [5]     | air-dried fruits/hydrodistillation Clevenger apparatus           |
| C     |                               | air-dried fruits/hydrodistillation Clevenger apparatus           |
| D     |                               | air-dried fruits/hydrodistillation Clevenger apparatus           |
| E     | Lis and Góra, 2001 [18]       | fresh flowers/hydrodistillation                                  |
| F     |                               | fresh leaves/hydrodistillation                                   |
| G     |                               | fresh crushed unripe fruits/hydrodistillation                    |
| H     |                               | fresh crushed ripe fruits/hydrodistillation                      |
| I     |                               | air-dried crushed ripe fruits/hydrodistillation                  |
| J     | Stoyanova et al., 2003 [17]   | air-dried crushed fruits stored for 0–6 months/hydrodistillation |
| K     |                               | air-dried crushed fruits stored for 6 months/hydrodistillation   |
| L     |                               | air-dried crushed fruits stored for 18 months/hydrodistillation  |
| M     |                               | air-dried crushed fruits stored for 30 months/hydrodistillation  |
| N     |                               | air-dried crushed fruits stored for 40 months/hydrodistillation  |
| O     | Chen et al., 2017 [16]        | air-dried crushed fruits/MHD-LLE *                               |
| P     |                               | air-dried crushed fruits/MHD-LLE **                              |
| Q     |                               | air-dried crushed fruits/microwave-assisted hydrodistillation    |
| R     |                               | air-dried crushed fruits/hydrodistillation Clevenger apparatus   |
| S     | Marinas et al., 2021 [22]     | air-dried crushed fruits/hydrodistillation Clevenger apparatus   |
| T     | Kozuharova et al., 2020 [23]  | air-dried fruits/hydrodistillation Clevenger apparatus           |

\* MHD-LLE—microwave-assisted hydrodistillation concatenated liquid–liquid extraction procedure—essential oil in first separation column; \*\* microwave-assisted hydrodistillation concatenated liquid–liquid extraction procedure—essential oil in second separation column.

Samples E and F (the essential oils of the flowers and leaves, respectively), as expected, statistically differed from any other sample. However, some observations were unexpected. Samples O, Q, and R from Chen et al. [16] ended up in the same cluster, but sample P was quite different, as shown by both clustering methods (Figures 1 and 2). The additional liquid–liquid separation column used for obtaining sample P (compared to sample O) seems to have significantly changed its composition. Sample S from Marinas et al. [22] is very closely related to the sample from Chen et al.

Based on these observations, sample A, obtained in this study, is not related to any of the previously reported samples. Although the analysis shows it is closest to samples B–D from Ivanescu et al. [5], germacrene D, which is the major constituent in our oil, was not detected in four samples from Romania. To better understand the differences between the obtained clusters, we also performed cluster characterization (post-clustering process where centroids or features within each cluster are analyzed to understand which variables are driving the formation of the clusters). For more details, see Cell 11 in *amorphia.html*, Supplementary Materials. With all this in mind, it is our opinion that the *A. fruticosa* used in this study represents a unique (germacrene D-driven) chemotype, which is distinctive from three other chemotypes:

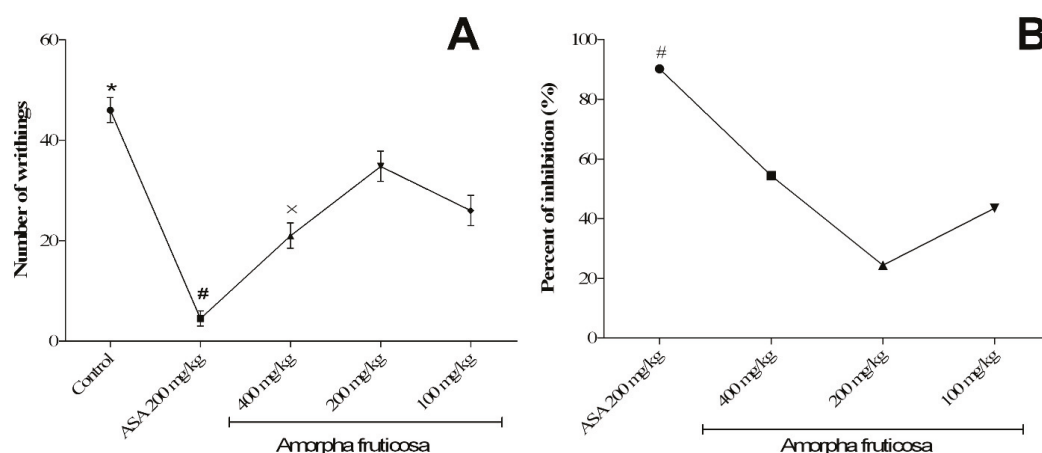
1. Monoterpene-dominated chemotype [18], major cluster contributors:  $\alpha$ -pinene (centroid value = 21.6) and myrcene (16.6);
2. Sesquiterpene-dominated chemotype I [16,17]; in our opinion, the *A. fruticosa* used in these two studies are of the same chemotype, and the difference in the clusters is caused only by different drying periods: samples K–N were obtained from fruit that was dried for over 6 months, while other samples were from fruit dried for a shorter period of time. Major cluster contributors:  $\delta$ -cadinene,  $\gamma$ -muurolene,  $\gamma$ -cadinene;
3. Sesquiterpene-dominated chemotype II [5], similar to chemotype A but with lower levels of  $\delta$ - and  $\gamma$ -cadinene and higher contribution of  $\alpha$ -zingiberene and  $\alpha$ -eudesmol.

The results of the clustering analysis supported our decision to use fresh *A. fruticosa* fruits in this study. Stoyanova et al. [17] reported that the volatile profile of this plant species varies depending on the storage period, and this effect can be clearly observed in the PCA scatter plot (Figure 2), where the essential oil from samples stored for longer periods (>6 months, K, L, M, N) forms a distinct cluster compared to the oil from fresh fruits (J). Thus, we chose to use essential oil from fresh fruits for two main reasons:

- (1) The profile of fresh material closely reflects the state of the plant at the time of collection. This is especially important for future studies dealing with the discovery of new chemotypes.
- (2) Another key reason was to ensure the reproducibility of the study. Any biological activity observed for *A. fruticosa* essential oil would be more reproducible when fresh fruit is used. Storage or drying introduces variability (e.g., loss of monoterpenes, oxidation), which is difficult to control. Even attempts to replicate the storage process would need to account for factors such as temperature and humidity. These variables are eliminated when fresh material is used.

### 2.3. Antinociceptive Activity

The essential oil demonstrated strong-to-moderate antinociceptive activity in the chemically induced pain model (Figure 3). A statistically significant difference in the degree of activity was found between the groups of animals treated with all doses of the essential on one side and vehicle/ASA-treated animals on the other ( $p < 0.01$ ). Additionally, the calculated percentage of abdominal writhing inhibition for the 400 mg/kg dose of the oil (54.4%) statistically differed from the positive control, aspirin (90.2%).



**Figure 3.** Effect of acute administration of *A. fruticosa* essential oil in doses of 100, 200, and 400 mg/kg i.p. on the number of writhes in mice induced by acetic acid. Values are mean  $\pm$  SD, one-way ANOVA followed by Tukey's test; \*  $p < 0.01$  vs. ASA and essential oil in all doses; #  $p < 0.01$  vs. vehicle and essential oil in all doses;  $\times$   $p < 0.01$  vs. *A. fruticosa* (100 and 200 mg/kg). (A): number of writhings vs. concentration; (B): percent of inhibition vs. concentration.

The injection of acetic acid triggers the release of prostaglandins, such as  $\text{PGE}_{2\alpha}$  and  $\text{PGF}_{2\alpha}$ , along with sympathetic nervous system mediators in the peritoneal fluid, causing a cascade of events that lead to abdominal contortions due to inflammation [24]. This test is nonspecific, since numerous drugs acting on different receptor/enzyme systems can reduce the number of acetic acid-induced writhes [25]. Amorfrutin A, a natural compound found in *A. fruticosa* fruits, has been shown to act as a nuclear factor- $\kappa$ B inhibitor (via different mechanisms), providing evidence for its potential usage in pathological conditions that involve inflammation. However, we did not detect it and it is very unlikely that it could be an essential oil component [26].

Our essential oil possessed significant amounts of  $\alpha$ -pinene (>4.2%), which was proven to significantly reduce the number of writhes induced by acetic acid in a dose

of 200 mg/kg [27]. However, several other constituents, such as germacrene D, caryophyllene, zingiberene,  $\delta$ - and  $\gamma$ -cadinenes, and others (Table 1) may also contribute to the overall activity of the essential oil.

### 3. Conclusions

The GC and GC-MS analyses of *A. fruticosa* fruit essential oil from Serbia led to the identification of 105 constituents, with germacrene D being the major compound. Clustering analysis suggests that the *A. fruticosa* used in this study represents a distinct new (germacrene D-dominated) chemotype and differs from the three previously reported chemotypes (one monoterpenoid-dominated and two sesquiterpenoid-dominated types). The statistical analysis also confirmed that fruit storage can affect essential oil composition, and prolonged storage could even result in the false identification of new chemotypes.

For this reason, we believe future studies on *A. fruticosa* essential oil should focus on using fresh plant material to ensure the reproducibility of biological assays, as the oil composition directly influences the outcomes of these assays. Changes in oil composition due to storage could affect the biological activities. Additionally, the hydrodistillation of fresh material prevents the loss of volatile compounds and ensures higher oil yields. Future research should also explore whether different hydrodistillation methods (e.g., microwave-assisted hydrodistillation, CO<sub>2</sub> extraction, etc.) can further increase yields.

The essential oil exhibited strong-to-moderate antinociceptive activity in a chemically induced pain model. Given the high yield of oil and the wide geographical distribution of this plant species, we believe that *A. fruticosa* essential oil has significant phytopharmacological potential. Further research is needed to identify the compounds responsible for this activity.

### 4. Materials and Methods

#### 4.1. GC and GC-MS

The GC-MS analyses were performed on a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5% diphenylsiloxane, 95% dimethylsiloxane, 30 m  $\times$  0.25 mm, film thickness 0.25  $\mu$ m; Agilent Technologies, Palo Alto, CA, USA) and coupled with a 5975B mass-selective detector from the same company. The injector and interface temperatures were set to 250 °C and 320 °C, respectively. The oven temperature was programmed to increase from 70 to 290 °C at a rate of 5 °C/min, followed by an isothermal hold for 10 min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. A sample (10  $\mu$ L of the oil in 1 mL Et<sub>2</sub>O) was injected in pulsed-split mode (with a flow rate of 1.5 mL/min for the first 0.5 min, then 1.0 mL/min for the remainder of the analysis; split ratio 40:1). The MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range of 35–500 amu, and a scan time of 0.34 s. The GC (FID) analyses were performed under the same experimental conditions using the same column as described for GC-MS. The percentage composition was calculated from the GC peak areas without the use of correction factors. Linear retention indices were calculated for all identified components using standards of *n*-alkanes (C<sub>7</sub>–C<sub>17</sub>). AMDIS (v. 2.70) software was used for chromatogram deconvolution and mass spectral libraries (Wiley 7, NIST 14, MassFinder 2.3, and Adams library [28]) were searched with NIST MS Search software (v. 2.0).

#### 4.2. Plant Material

Plant material (ripe fruits) of *A. fruticosa* was collected in September 2011 near the Nišava River in Niš, Serbia (exact coordinates: 43°19'13" N, 21°56'27" E). A voucher specimen was deposited with the Herbarium Collection of the Faculty of Sciences and Mathematics, University of Niš, under acquisition number 18661. The identity of the material was confirmed by the curator of the herbarium.

#### 4.3. Chemicals

All solvents (HPLC-grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Authentic chemical samples were obtained from Merck (Darmstadt, Germany), and Carl Roth (Karlsruhe, Germany) in the highest available purity. For the determination of retention indices, a commercial *n*-alkane mixture (Sigma-Aldrich, St. Louis, MO, USA) ranging from heptane to eicosane (C<sub>7</sub>–C<sub>40</sub>) was used.

#### 4.4. Extraction of Essential Oils

Fresh fruits (260 g) of *A. fruticosa* were homogenized in a blender and submitted to hydrodistillation with approximately 2.5 L of H<sub>2</sub>O for 3 h, using an original Clevenger-type apparatus. The obtained oil was separated by extraction with Et<sub>2</sub>O and dried with anhydrous MgSO<sub>4</sub>. The solvent was evaporated under a gentle stream of N<sub>2</sub> at room temperature, to exclude any loss of the essential oil, and the sample was immediately analyzed. To determine the oil yield, after most of the Et<sub>2</sub>O was removed under a stream of N<sub>2</sub>, the residue was exposed to a vacuum at room temperature for a short time to eliminate any remaining solvent. The yield of the obtained oil (3.5 g) was 1.3% (*w/w*). The essential oil was stored in a freezer at −20 °C without any further treatment.

#### 4.5. Clustering Analysis

The dataset used for the statistical analysis (*amorpha\_fruticosa\_dataset.csv*, Supplementary Materials) was compiled from composition data for essential oils labeled A–Q (see Table 2 for the origin of each sample). Constituents that occurred above the 1% level were filtered and used for the analysis. All analyses were performed using the scikit-learn package in Python 3.10.13 [29] within the Jupyter Lab (v. 4.0.9) environment [30]. Visualization was performed using the matplotlib and Plotly packages. The complete code is provided in the Supplementary Materials in both .ipynb and .html formats.

Prior to analysis, the dataset was transposed to facilitate clustering. Hierarchical clustering was conducted using agglomerative clustering with Euclidean distance as the metric. The pairwise distance matrix was computed using the *pdist* function, and hierarchical clustering was performed using the Ward method to generate a linkage matrix. A dendrogram was created to visualize the hierarchical clustering, with a color threshold set at 25. Based on this dendrogram, eight clusters were selected for agglomerative clustering. Samples within each cluster were identified and listed.

Principal component analysis (PCA) was used to reduce the dimensionality of the dataset to two principal components. *K*-means clustering was applied to the PCA-transformed data. The optimal number of clusters was determined using the Elbow Method, which involved plotting the inertia values for a range of clusters (*K* = 1 to 10). Based on visual observation, eight clusters were selected for *k*-means clustering. The results were visualized using a scatter plot created with Plotly Express (v. 5.24.1) [31], where the two PCA components were plotted, and points were colored according to their cluster assignment.

#### 4.6. Animals and Treatment

The adult male and female Wistar rats (200–250 g) used in this experiment were housed in our facilities (Vivarium of the Institute of Biomedical Research, Medical Faculty, University of Niš, Niš, Serbia). They were kept in groups of 6 animals per cage, maintained in standard laboratory conditions at 22 ± 2 °C and 60% humidity, with food and water available *ad libitum*. All animals fasted for 12 h, although they were still allowed free access to water, before the commencement of the experiments. All rats were individually weighed, and the essential oil of *A. fruticosa* L. was applied at doses of 400, 200, and 100 mg/kg dissolved in olive oil, while the negative and positive control groups received the vehicle (olive oil in a dose of 10 mL/kg) and aspirin (ASA; in a dose of 200 mg/kg), respectively. All substances were administered intraperitoneally (i.p.) to animals 1 h before the test.

The experiments were performed in accordance with the Declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU



Directive of 2010; 2010/63/EU), and the experimental protocols were commenced after being approved by the institutional animal ethics committee (No. 01-4097-2 from 2011).

#### 4.7. Acetic Acid-Induced Abdominal Writhing

The method adopted in this study was previously described by Radulović et al. [24]. Briefly, one hour after the administration of all substances, 1% (*v/v*) acetic acid (500 µL) was injected i.p. into all animals. During the next 20 min, specific contractions of the abdominal muscles, described as writhing, were counted. The % of inhibition was calculated as follows:

$$\% \text{ inhibition} = 100 \times \frac{\text{number of writhes (control)} - \text{number of writhes (test)}}{\text{number of writhes (control)}}$$

#### 4.8. Statistical Analysis

Data obtained from the experiments are presented as mean ± SD and were further analyzed using one-way ANOVA followed by Tukey's post hoc test (GraphPad Prism version 5.03, San Diego, CA, USA). Probability values (*p*) less than 0.05 were considered statistically significant.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants13213045/s1>, *amorpha\_fruticosa\_dataset.csv* (dataset used for the clustering analysis), *amorpha.ipynb* (Jupyter notebook with Python code), and *amorpha.html* (*amorpha.ipynb* converted to .html format so it can be viewed in a browser).

**Author Contributions:** Conceptualization, N.S.R. and N.M.S.; methodology, N.S.R., N.M.S. and P.J.R.; software, D.B.Z.; formal analysis, M.R.Đ.Z., N.M.S. and D.B.Z.; investigation, M.R.Đ.Z., N.M.S. and D.B.Z.; resources, N.S.R.; data curation, D.B.Z.; writing—original draft preparation, M.R.Đ.Z., N.M.S. and D.B.Z.; writing—review and editing, N.S.R. and P.J.R.; supervision, N.S.R.; project administration, N.M.S.; funding acquisition, N.S.R. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Data are contained within the article and Supplementary Materials.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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## Article

# Assessing a “Least-Concern” Red List Tree Species from Madagascar Used in Traditional Medicine: *Morella spathulata* (Myricaceae) Phyto-Compounds and Anti-Inflammatory Properties

Annachiara Fioccardi <sup>1,\*</sup>, Dario Donno <sup>1</sup>, Zoarilala Rinah Razafindrakoto <sup>2</sup>, Nantenaina Tombozara <sup>2</sup>, Sylvia Henintsoa <sup>1</sup>, Elyna Mahitasoa <sup>2</sup>, Valeria Torti <sup>3</sup>, Marcellin Solofoniaina <sup>2</sup>, Lorenzo Rosso <sup>1</sup>, Giovanni Gamba <sup>1</sup>, Charles Andrianjara <sup>2</sup>, David Ramanitrahassimbola <sup>2</sup> and Gabriele Loris Beccaro <sup>1</sup>

<sup>1</sup> Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, 10095 Grugliasco, TO, Italy; dario.donno@unito.it (D.D.); hnsylvia98@gmail.com (S.H.); lorenzo.rosso@unito.it (L.R.); giovanni.gamba@unito.it (G.G.); gabriele.beccaro@unito.it (G.L.B.)

<sup>2</sup> Institut Malgache de Recherches Appliquées, B.P. 3833, Antananarivo 101, Madagascar; zo\_ari\_lala@yahoo.fr (Z.R.R.); nzara89@gmail.com (N.T.); elynamahitasoa@gmail.com (E.M.); yvesmickaelsolofoniaina@gmail.com (M.S.); charles.andrianjara@gmail.com (C.A.); ramanitrahassimboladavid@gmail.com (D.R.)

<sup>3</sup> Dipartimento Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, 10123 Torino, TO, Italy; valeria.torti@unito.it

\* Correspondence: annachiara.fioccardi@unito.it; Tel.: +39-3476623441

**Abstract:** *Morella spathulata* (Myricaceae family) is a common plant from Madagascar and is present on the IUCN Red List of threatened species classified at the ‘least concern’ level, used by the local population to treat numerous illnesses and pain. Despite its frequent use, comprehensive phytochemical and pharmacological research on the species is limited. This study evaluated the antioxidant, analgesic, and anti-inflammatory properties, as well as the toxicity of methanol extracts from the leaves (MS\_L) and bark (MS\_B) of *M. spathulata*. The research involved the analysis of nutritional traits such as sugars, organic acids, vitamin C, polyphenolic content (TPC) and the main phytochemicals by HPLC analysis. Antioxidant capacity was assessed through DPPH and FRAP assays. Analgesic and anti-inflammatory activities were evaluated using acetic acid-induced writhing and carrageenan-induced paw oedema tests in mice. The results showed a high content of phenolic and bioactive components in the leaf and bark extracts, associated with antioxidant, analgesic and anti-inflammatory properties. The interaction of key compounds such as ferulic acid and ellagic acid with proteins involved in pH regulation and immune modulation provides clues to the mechanisms underlying the therapeutic effects. However, conservation efforts are crucial due to habitat loss and illegal logging, and further studies are needed to fully explore the plant’s therapeutic potential.

**Keywords:** *Morella spathulata*; antioxidant; traditional medicine; analgesic activity; anti-inflammatory effects; phytochemicals

## 1. Introduction

The application of medicinal plants in the traditional African healthcare system is one of the oldest and most diverse therapeutic approaches. Throughout history, traditional medicine has consistently served as an alternative solution for various illnesses [1]. More than 90% of the world’s population uses traditional medicines based on natural products as primary health care. Among the actual pharmaceutical products and drugs, almost 73% are derived from natural products, including active substances extracted from plants [2]. Especially in developing countries, such as Madagascar, where access to modern medical resources is often limited or financially expensive, the use of health-promoting remedies

derived from natural resources is very important [3]. In addition, the deep-rooted cultural familiarity, traditionalism, and perceived safety of traditional medicine are influential, making it the preferred choice among the local population [4,5].

Madagascar has an interesting natural heritage of medicinal plants, which contribute to its rich pharmacopoeia, thanks to its wide range of plant species. The highly abundant species is a result of Madagascar's detachment from Africa approximately 170 million years ago, and then by the Indian subcontinent nearly 88 million years ago, making it the largest reserve of endemism and biodiversity on the planet [4,6]. This botanical diversity is a valuable resource for traditional and modern medicine. In Malagasy traditional medicine, several plants are used to cure gastrointestinal disorders such as diarrhea and intestinal parasites [4]. Furthermore, this conventional practice is also applied to the prevention of respiratory infections like bronchitis and colds, which are common in rural areas of the country [7]. This traditional practice becomes increasingly important in the context of rising drug costs and resistance to diseases, including malaria, bacterial infections, and sexually transmitted diseases. As a result, there is a new focus on exploring traditional medicine as a promising strategy for the discovery of new chemical compounds against these health challenges [8]. However, the spontaneous collection of medicinal plants in forests causes a negative impact on the local ecosystem, resulting in a significant reduction in biodiversity [9]. Nonetheless, the controlled propagation of these plant species presents a promising solution to replace the random collection of plants [10]. Propagation and dedicated cultivation can reduce overharvesting and ensure high-quality material production, emphasizing the importance of characterizing the Malagasy plant biodiversity by phytochemical and pharmacological analysis.

*Morella spathulata* (Mirb.) Verdc. and Polhill (Myricaceae family), also known as *Myrica spathulata* Mirb, is widespread across eastern Madagascar, from north to south provinces [11]. It is also present in Mozambique (in the Sofala region, approximately 90 km north of Beira) and Tanzania (Pemba), up to 1000 m above sea level [12], even if the botanical classification in this region is still unclear. *M. spathulata* is a shrub to small tree up to 12 m high. It occurs in grasslands with marginal or high-water tables on peaty soils, riverbanks, and sandy coast plains, frequently associated with *Erica* spp. [13,14]. The leaves are oblanceolate to strictly oblong-elliptical, closely obovate or obovate-spatulate, 2.5 to 9 cm long and 1.5 to 3.5 cm wide, rounded or sometimes notched at the apex, attenuate-decurrent at the base, leathery, smooth, and often shiny above, with small thick, consistent red-brown scales below, with 10 to 20 lateral veins on all side, rather inconspicuous below. The inflorescences are glandular, oval, and obtuse, varying in length from 1.5 to 3 cm. The fruits are ovoid and globose, 4 to 5 mm in diameter [15]. This species represents an example of the exploitation of natural resources and loss of biodiversity. Indeed, although *M. spathulata* is distributed and present in protected areas, it is threatened by illegal logging, agriculture, and the slash-and-burn practice. Currently listed as 'least concern', its importance in traditional medicine is significant [16]. The leaves and barks of this species are prepared as infusion or decoction for various medicinal purposes on the east coast of Madagascar, including a mouthwash to relieve toothache, pain, and inflammation occurring during the sickle cell patient crisis [12]; the leaves were chewed and applied to wounds. Despite its medicinal virtues, this species has not yet been subjected to comprehensive phytochemical or pharmacological investigations. Therefore, the objective of this survey is to examine the antioxidant, analgesic, and anti-inflammatory activities, and acute toxicity, along with the phytochemical and nutritional traits of *M. spathulata* aerial parts extracts.

## 2. Results and Discussion

### 2.1. Total Polyphenolic Contents (TPC)

Plant phenols are a notable category of compounds known for their role as primary antioxidants or free radical terminators. Indeed, assessing their cumulative amount is a valuable tool for discerning plants and extracts with potential commercial prospects [17].

Furthermore, in several studies, as well as in the present work, the spectrophotometric method for TPC evaluation has been used in association with chromatographic analysis to strengthen and validate the results, establishing its credibility as a complementary technique [18,19]. In this study, TPC for *Morella spathulata* extracts ranged from  $8913.58 \pm 1940.88$  mg GAE/100 g DW in leaves to  $10,177.81 \pm 1241.09$  mg GAE/100 g DW in bark. These values are very high if compared to other species, such as *Morella parvifolia* (Benth.) Parra-Os., native to South America; in a study conducted by Puertas-Mejía, M. A. et al. [20], they identified a TPC value of  $51.41 \pm 0.44$  mg GAE/100 g DW in the leaves of *M. parvifolia*. The bark also showed a high value of TPC ( $10,177.81 \pm 1241.09$  mg GAE/100 g DW), which may be related to the high accumulation of polyphenolic components in bark tissues during the lignification processes, as confirmed by many studies [21,22].

## 2.2. Phytochemical Fingerprint

In this paper, 24 bioactive components and nutrients were chosen for their relevance in human health [23]. These compounds were used as markers to determine the HPLC fingerprint (Table 1). In general, the number of identified bioactive compounds was higher in the leaves than in the bark (eight vs. seven). Unlike spectrophotometric techniques, liquid chromatography offers the possibility of quantifying specific polyphenolic targets. Several markers, known for their high biological activity, were selected, and their higher presence in leaves could be due to the leaf structural differences compared to bark, which may present a lower concentration of these compounds. The value of bioactive substances with high antioxidant capacity in leaves and bark was examined by grouping and comparing the amount of the selected phytochemicals: polyphenolic components (flavonols, benzoic acids, cinnamic acids, and catechins) and vitamin C (dehydroascorbic and ascorbic acids) (Figure 1). Regarding cinnamic acids, leaf extract was the most significant contributor, with a mean value of 67.16%, as opposed to the bark, with a value of 49.67%. Flavonols, in leaves and bark extract contributed to the antioxidant compounds almost equally (with values of 2.63% and 2.50%, respectively). Benzoic acids were most abundant in bark extract (22.11%) rather than in leaves (19.05%). Vitamin C showed the highest value in leaf extract with a value of 14.49%.

**Table 1.** Phytochemical fingerprint of the analyzed samples.

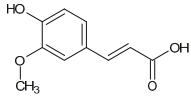
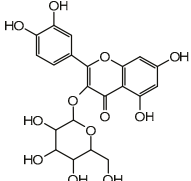
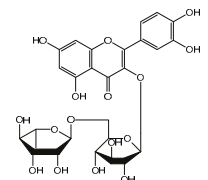
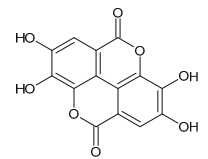
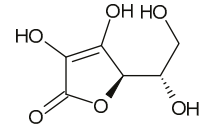
| Class          | Bioactive Marker | MS_L                                 | MS_B                                 | Identified Compounds:<br>Chemical Structures                                          |
|----------------|------------------|--------------------------------------|--------------------------------------|---------------------------------------------------------------------------------------|
|                |                  | Mean value $\pm$ SD<br>(mg/100 g DW) | Mean value $\pm$ SD<br>(mg/100 g DW) |                                                                                       |
| Cinnamic acids | caffeic acid     | n.d.                                 | n.q.                                 |  |
|                | chlorogenic acid | n.d.                                 | n.d.                                 |                                                                                       |
|                | coumaric acid    | n.q.                                 | n.d.                                 |                                                                                       |
|                | ferulic acid     | $67.16 \pm 0.73^a$                   | $49.67 \pm 2.09^b$                   |                                                                                       |
| Flavonols      | hyperoside       | n.q.                                 | n.d.                                 |  |
|                | isoquercitrin    | n.d.                                 | $2.5 \pm 0.46$                       |                                                                                       |
|                | quercetin        | n.d.                                 | n.d.                                 |                                                                                       |
|                | quercitrin       | n.d.                                 | n.d.                                 |                                                                                       |



Table 1. Cont.

| Class         | Bioactive Marker     | MS_L                     | MS_B                      | Identified Compounds:<br>Chemical Structures                                        |
|---------------|----------------------|--------------------------|---------------------------|-------------------------------------------------------------------------------------|
| Benzoic acids | rutin                | 2.63 ± 1.66              | n.d.                      |  |
|               | ellagic acid         | 19.08 ± 1.5 <sup>a</sup> | 22.11 ± 2.26 <sup>a</sup> |  |
|               | gallic acid          | n.d.                     | n.d.                      |                                                                                     |
| Catechins     | catechin             | n.d.                     | n.d.                      |                                                                                     |
|               | epicatechin          | n.d.                     | n.d.                      |                                                                                     |
| Vitamins      | ascorbic acid        | 14.49 ± 0.19             | n.d.                      |  |
|               | dehydroascorbic acid | n.d.                     | n.d.                      |                                                                                     |

MS\_L: *M. spathulata* leaf extract; MS\_B: *M. spathulata* bark extract. Each value represents the mean ± SD (standard deviation) (N = 3). Significant statistical differences (*p* < 0.05) are highlighted by different letters (a,b). DW = dried weight, n.d. = not detected, n.q. = not quantified.

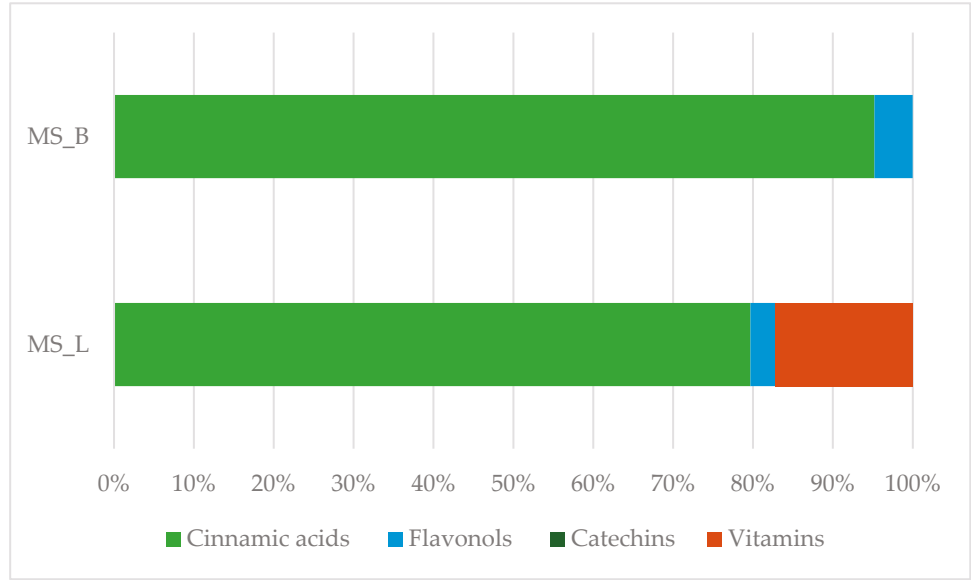


Figure 1. Percentage contribution of phenolic compounds and vitamins in the analyzed samples. MS\_L: *M. spathulata* leaf extract; MS\_B: *M. spathulata* bark extract. Mean values are shown (N = 3).

More in detail, within the class of cinnamic acids, the only compound quantified was ferulic acid, with a value of 67.16 ± 0.73 mg/100 g DW in the leaves and 49.67 ± 2.09 mg/100 g DW in the bark. Ferulic acid is known for its antimicrobial and antioxidant properties [24,25]. These properties may support its potential use in traditional medicine, especially considering the important quantities found. In the class of flavonols, only isoquercitrin was quantified in the bark (2.5 ± 0.46 mg/100 g DW) and only rutin was detected in the leaves with a value of 2.63 ± 1.66 mg/100 g DW. Among the class of benzoic acids,

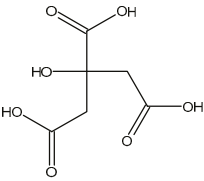
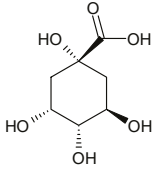
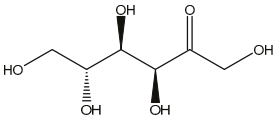
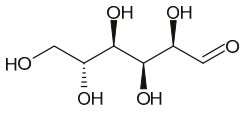
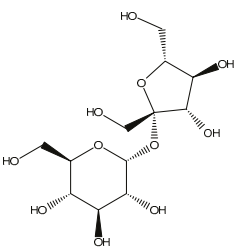
only ellagic acid was quantified with a value of  $19.08 \pm 1.5$  mg/100 g DW in the leaf and  $22.11 \pm 2.26$  mg/100 g DW in the bark. Plants produce ellagic acid by the hydrolysis of tannins [26]. Tannins concentration is generally greater in the bark compared to other parts of the plant [27], which likely accounts for the higher levels of ellagic acid that have been quantified in the bark. The ellagic acid content found in the leaves was similar to other methanolic extracts of endemic Malagasy plants (e.g., *Tina striata* with a value of  $19.33 \pm 2.96$  mg/100 g DW [28], and *Phyllarthron bojeranum* with a value of  $18.16 \pm 6.5$  mg/100 g DW [29]). The same is also true for the bark value ( $22.11 \pm 2.26$  mg/100 g DW), which can be compared to that reported in *Schefflera bojeri* ( $24.46 \pm 10.54$  mg/100 g DW) [30]. Ellagic acid plays a key role in anti-atherogenic, anti-inflammatory, and neuroprotective actions. In addition to its function as a liver and skin protector and as an anti-cancer agent, it influences cellular processes and DNA damage protection and has anti-inflammatory properties [31]. Furthermore, a positive correlation ( $R = 0.998$ ;  $p < 0.05$ ) was noted between ellagic and ferulic acids, suggesting a potential interaction between these two compounds in *M. spathulata*. This correlation indicates the complex biochemical relationships throughout the plant, which might affect their synergistic effects or shared metabolic pathways.

To further investigate the correlation found between ellagic acid and ferulic acid, the identification of their molecular targets and pharmacokinetic profiles was carried out using the Swiss Target Prediction platform (<http://www.swisstargetprediction.ch/>, accessed on 8 April 2024), as previously reported in Fawzi M. et al. [32]. Based on the platform, it was predicted that the targets of ferulic acid are carbonic anhydrases (CA2), a key enzyme for acid–base balance and carbon dioxide transport [33]. CA2, present in tissues such as the liver, also plays a crucial role in pH regulation [34]. The activity of CA2 has an impact on conditions related to acid–base imbalance, and its interaction with ferulic acid may suggest potential anti-inflammatory effects [35]. In comparison, ellagic acid was found linked to the G protein-coupled receptor 35 (GPR35). It is present in various organs, among them the digestive tract and immune cells [36], and the interaction of ellagic acid with GPR35 may influence immune regulation and inflammation, contributing to anti-inflammatory and neuroprotective properties. Moreover, the role of GPR35 in gastrointestinal health also suggests a potential impact on digestive functions [37,38]. Catechins have not been detected. Many studies confirmed that catechins are highly unstable in sunlight conditions [39,40]. In this case, plant materials of *M. spathulata* were dried in sunlight according to local traditions, which may have influenced the degradation of catechins. About vitamin C, ascorbic and dehydroascorbic acids were evaluated. In this study on *Morella spathulata*, only ascorbic acid was found in the leaves with a value of  $14.49 \pm 0.19$  mg/100 g DW. Vitamin C content varies depending on species and environmental conditions and among different meristematic tissues and organs, generally being high in flowers, leaves, and young fruits and low in non-photosynthetic tissues such as stems, roots, and bark [41,42]. For this reason, vitamin C may have been only identified in the leaves and not in the bark. Vitamin C functions as an antioxidant safeguarding the body from oxidative stress and preserving the immune system [43]. Additionally, it has the capability of neutralizing radicals and damaged lipids and DNA in the blood, which can lead to disease [44].

Organic acids and sugars are reported in Table 2. Both in plant materials of *M. spathulata*, quinic acid was identified; a value of  $48.57 \pm 0.68$  g/100 g DW was detected in the leaves and  $21.50 \pm 0.75$  g/100 g DW in the bark. In vitro and in vivo pharmacological studies have demonstrated that quinic acid possesses several biological activities, including antioxidant, antitumor, antimicrobial, antiviral, and analgesic properties [45]. The antioxidant activity of this molecule was also confirmed by the correlation ( $R = 0.999$ ;  $p < 0.05$ ) with antioxidant capacity (AOC). In addition, it stimulates insulin secretion by releasing intracellular  $\text{Ca}^{2+}$  and increasing the NAD(P)H/NAD(P)<sup>+</sup> ratio, contributing to anti-diabetic effects [46]. Instead, citric acid was identified in the bark extract with a value of  $3.55 \pm 0.46$  g/100 g DW. Citric acid is an excellent harmless disinfectant against various viruses [47]. Its presence may validate the use of *M. spathulata* in traditional medicine as a wound disinfectant. In addition, citric acid was indicated as a potent antioxidant [48],

confirmed by the positive correlation ( $R = 0.997$ ) with the values of free radical scavenging activity (DPPH).

Table 2. Organic acids and sugars of the analyzed samples.

| Class.        | Bioactive Marker               | MS_L                                | MS_B                                | Identified Compounds:<br>Chemical Structures                                          |
|---------------|--------------------------------|-------------------------------------|-------------------------------------|---------------------------------------------------------------------------------------|
|               |                                | Mean value $\pm$ SD<br>(g/100 g DW) | Mean value $\pm$ SD<br>(g/100 g DW) |                                                                                       |
| Organic acids | citric acid                    | n.d.                                | $3.55 \pm 0.46$                     |    |
|               | malic acid                     | n.d.                                | n.d.                                |                                                                                       |
|               | oxalic acid                    | n.d.                                | n.d.                                |                                                                                       |
|               | quinic acid                    | $48.57 \pm 0.68^a$                  | $21.50 \pm 0.75^b$                  |    |
|               | succinic acid<br>tartaric acid | n.d.<br>n.d.                        | n.d.<br>n.d.                        |                                                                                       |
| Sugars        | fructose                       | $1.26 \pm 0.04^b$                   | $1.75 \pm 0.03^a$                   |  |
|               | glucose                        | $24.81 \pm 0.76^b$                  | $31.44 \pm 1.22^a$                  |  |
|               | sucrose                        | $6.04 \pm 0.54$                     | n.d.                                |  |

MS\_L: *M. spathulata* leaf extract; MS\_B: *M. spathulata* bark extract. Each value represents the mean  $\pm$  SD (standard deviation) ( $N = 3$ ). Significant statistical differences ( $p < 0.05$ ) are highlighted by different letters (a,b). DW = dried weight, n.d. = not detected.

*M. spathulata* showed the highest sugar content in the leaves and bark for glucose ( $24.81 \pm 0.76$  g/100 g DW and  $31.44 \pm 1.22$  g/100 g DW, respectively). Compared to other Malagasy plants studied, which had glucose values below 1 g/100 g DW [28], a significantly higher glucose content was observed here. The high glucose content quantified in this plant could be beneficial for diabetics in hypoglycemic situations, representing a source of sugar to consume when necessary. Furthermore, according to the Swiss Target Prediction platform (<http://www.swisstargetprediction.ch/>, accessed on 8 April 2024), the glucose molecule was identified to target the protein Cholinergic Receptor Nicotinic Alpha 7 Subunit (CHRNA7), a receptor crucial for the anti-inflammatory cholinergic pathway associated with autoimmune disorders [49]. It also contributes to cell proliferation and angiogenesis in lung and pancreatic cancers and serves as a key component of the cholinergic nervous system in the brain [50,51]. Fructose was detected in similar levels both in

leaf and bark extracts, with values of  $1.26 \pm 0.04$  g/100 g DW and  $1.75 \pm 0.03$  g/100 g DW, respectively. It is interesting to note that fructose can act as an antioxidant due to its reducing capacity [52]. Sucrose was only detected in leaf extract with a value of  $6.04 \pm 0.54$  g/100 g DW, as it is one of the main products of photosynthesis in leaves [53]. In general, contrary to other Malagasy flora, which are generally characterized by a lower carbohydrate content, *M. spathulata* could be a promising food resource, especially in countries with high malnutrition rates, due to its considerable sugar levels.

### 2.3. In Vitro Antioxidant Capacity (DPPH and FRAP)

In *M. spathulata* bark and leaves, the antioxidant properties were evaluated by two different tests: the free radical scavenging activity test (DPPH) and the ferric reducing antioxidant power test (FRAP). All the results of the antioxidant capacity are shown in Table 3. The DPPH is a rapid and simple method to test antioxidant activity based on the ability of some molecules to perform their capacity of being radical scavengers and hydrogen donors. In this case, the scavenging potential of extracts is based on the DPPH solution discoloration from purple (free radical stable in methanol) to yellow (DPPH reduced by radical scavenger) [54].

**Table 3.** FRAP, and IC<sub>50</sub> values of MS\_L and MS\_B by DPPH assay.

| Sample      | FRAP (mmol Fe <sup>2+</sup> /kg DW) | Yield (%) | Equation               | r <sup>2</sup> | DPPH Assay: IC <sub>50</sub> (µg/mL) |
|-------------|-------------------------------------|-----------|------------------------|----------------|--------------------------------------|
| MS_L        | $227.89 \pm 12.53^a$                | 3.90      | $y = 2.0955x - 6.1227$ | 0.9994         | $26.78 \pm 0.42^a$                   |
| MS_B        | $376.18 \pm 115.69^a$               | 9.84      | $y = 2.0098x - 9.7684$ | 0.9999         | $29.74 \pm 0.16^a$                   |
| Gallic acid | -                                   | -         | $y = 2.7242x - 9.3331$ | 0.9989         | $21.78 \pm 0.11$                     |

MS\_L: *M. spathulata* leaf extract; MS\_B: *M. spathulata* bark extract; DW: dried weight. Each value represents the mean  $\pm$  SD (standard deviation) (N = 3), <sup>a</sup>:  $p < 0.05$  vs. gallic acid.

As shown in Table 3, maceration yielded 9.84% of MS\_B and 3.90% of MS\_L. The IC<sub>50</sub> values were  $26.78 \pm 0.42$  mg/mL for MS\_L and  $29.74 \pm 0.16$  mg/mL for MS\_B. These values were close to those of gallic acid (IC<sub>50</sub> =  $21.78 \pm 0.11$  mg/mL), which represented the positive control used. MS\_L and MS\_B were more active compared to other Malagasy medicinal plants as reported in previous studies. For example, the aerial parts of *Vaccinium secundiflorum* were characterized by an IC<sub>50</sub> value of  $76.07 \pm 1.08$  mg/mL [55], while the extracts of *Uapaca bojeri* showed lower IC<sub>50</sub> values ( $47.36 \pm 3.00$  and  $33.32 \pm 0.69$  mg/mL, respectively, for leaves and bark) [54]. The *Lygodium lanceolatum* and *Imperata cylindrica* aerial parts presented IC<sub>50</sub> of  $107.05 \pm 3.41$  mg/mL and IC<sub>50</sub> of  $192.07 \pm 0.78$  µg/mL, respectively [56,57]. The DPPH values on *M. spathulata* antioxidant capacity were also confirmed by FRAP results. The FRAP values were  $227.89 \pm 12.53$  mmol Fe<sup>2+</sup>/kg of DW for the leaves and  $376.18 \pm 115.69$  mmol Fe<sup>2+</sup>/kg of DW for the bark. Such values are higher than those found in another Malagasy medicinal plant, *Uapaca bojeri*, where the leaves and bark had values of  $69.20 \pm 1.41$  mmol Fe<sup>2+</sup>/kg of DW and  $70.17 \pm 9.53$  mmol Fe<sup>2+</sup>/kg of DW, respectively [54]. These results confirmed the importance of *M. spathulata* in popular tradition as a source of potentially beneficial treatments and underlined the necessity of conserving this plant resource.

### 2.4. Anti-Inflammatory and Analgesic Activity

#### 2.4.1. Carrageenan-Induced Paw Oedema

Reducing oedema rates exerted by MS\_L and MS\_B are reported in Table 4. The *M. spathulata* extracts showed significant ( $p < 0.05$ ) oedema reductions, at all tested doses (100, 200 and 400 mg/kg b.w.) at every time of measurement (30, 60, 120, 180 and 240 min) in comparison with the negative control (vehicle) as is similar to indomethacin at the dosage of 10 mg/kg b.w. This oedema reduction also occurs in a dose-dependent way at 2 and 3 h ( $p < 0.001$  with ANOVA) after the induction of inflammation when mice were treated with both extracts. MS\_L and MS\_B (200 and 400 mg/kg,  $p < 0.05$ , respectively) showed

higher inhibition of paw oedema 2 and 3 h of post-carrageenan induction. Carrageenan is largely utilized as a phlogistic agent to lead to acute inflammation, so the carrageenan-induced paw oedema model is often employed as the method for testing non-steroidal anti-inflammatory (NSAID) agents [58,59]. This protocol is biphasic. The initial phase (1 to 1.5 h) is characterized by non-phagocytic oedema, succeeded by a secondary phase with increased oedema development occurring over the remaining five hours. Different mediators are involved in the various phases of carrageenan-induced oedema. The early stage (up to the first 1.5 h) is dominated by the release of histamine, serotonin, 5-hydroxytryptamine, and PAF (platelet-activating factor). Kinin is released from 1.5 to 2.5 h in the last phase, inflammation will persist to the fifth hour by lipid-derived eicosanoids (prostaglandin, leukotriene, eicosatetraenoic acid 5-hydroperoxide and others) [60,61].

**Table 4.** Effects of MS\_L and MS\_B on carrageenan-induced mouse paw oedema (n = 5).

| Sample       | Dose (mg/kg) | Inhibition of inflammation (%) |                             |                             |                             |                             |
|--------------|--------------|--------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|              |              | 30 min                         | 60 min                      | 120 min                     | 180 min                     | 240 min                     |
| Vehicle      | 0            | 4.28 ± 1.87                    | 9.47 ± 2.52                 | 18.72 ± 2.26                | 19.99 ± 5.45                | 32.95 ± 2.77                |
| MS_L         | 100          | 11.03 ± 3.21 <sup>a</sup>      | 22.59 ± 2.50 <sup>b</sup>   | 39.64 ± 3.15 <sup>b</sup>   | 62.32 ± 4.44 <sup>b</sup>   | 85.74 ± 2.49 <sup>b,c</sup> |
|              | 200          | 16.85 ± 5.51 <sup>b</sup>      | 32.76 ± 6.23 <sup>b,c</sup> | 65.33 ± 6.95 <sup>b,c</sup> | 75.71 ± 7.74 <sup>b,c</sup> | 88.68 ± 5.77 <sup>b,c</sup> |
|              | 400          | 11.89 ± 1.53 <sup>a</sup>      | 29.58 ± 6.84 <sup>b</sup>   | 52.44 ± 7.04 <sup>b</sup>   | 80.12 ± 6.42 <sup>b,c</sup> | 94.25 ± 1.78 <sup>b,c</sup> |
| MS_B         | 100          | 6.40 ± 1.15 <sup>a</sup>       | 17.43 ± 2.74 <sup>b</sup>   | 29.77 ± 2.13 <sup>b,c</sup> | 43.49 ± 3.43 <sup>b</sup>   | 49.88 ± 6.64 <sup>b,c</sup> |
|              | 200          | 12.95 ± 4.05 <sup>b</sup>      | 30.21 ± 5.29 <sup>b</sup>   | 59.70 ± 3.79 <sup>b</sup>   | 74.57 ± 5.19 <sup>b,c</sup> | 91.41 ± 3.57 <sup>b,c</sup> |
|              | 400          | 12.66 ± 2.83 <sup>b</sup>      | 31.58 ± 6.95 <sup>b</sup>   | 57.10 ± 8.76 <sup>b</sup>   | 85.65 ± 5.83 <sup>b,c</sup> | 96.53 ± 1.90 <sup>b,c</sup> |
| Indomethacin | 10           | 13.77 ± 3.89 <sup>b</sup>      | 24.02 ± 8.60 <sup>b</sup>   | 40.26 ± 7.87 <sup>b</sup>   | 57.59 ± 4.14 <sup>b</sup>   | 71.89 ± 1.84 <sup>b</sup>   |

Values are expressed as mean ± s.e.m of five independent experiments. <sup>a</sup>:  $p < 0.05$  vs. vehicle. <sup>b</sup>:  $p < 0.01$  vs. vehicle. <sup>c</sup>:  $p < 0.05$  vs. indomethacin.

*M. spathulata* extracts were active in both phases of inflammation, but the activities were more pronounced in the late phase. The highest activity was observed at a dose of 400 mg/kg, with inhibition percentages of 94.25% for MS\_L and 96.53% for MS\_B at 240 min. Indomethacin (10 mg/kg), a cyclooxygenase inhibitor, can mitigate the carrageenan reaction in the second stage of inflammation (71.89%, 240 min), mainly through prostaglandin mediation [62]. The anti-inflammatory effects of extracts, such as indomethacin, were noted especially in the second stage of inflammation. This might imply that the anti-inflammatory effect is caused by the inhibition of prostaglandin release. Nevertheless, the initial anti-inflammatory effects of MS\_L (29.58% at 400 mg/kg) and MS\_B (31.58% at 400 mg/kg) against carrageenan-induced inflammation, resulting from the release of histamine, serotonin and kinin-like substances in the initial period (1 h) may not be excluded. The anti-inflammatory property of this plant might be due to the content of polyphenols detected in the plant; indeed, this study revealed a positive correlation ( $R = 0.998$ ;  $p < 0.05$ ) between TPC and anti-inflammatory activity. Moreover, quinic acid, representing 26% and 16% of the total identified compounds in MS\_L and MS\_B, respectively, and its derivatives have been reported for their anti-inflammatory activities [63]. The mechanism by which quinic acid exerts its anti-inflammatory activity is uncertain, but it appears to be linked to the inhibition of the proinflammatory nuclear factor kappa B (NF- $\kappa$ B) [64,65] contributing to the anti-inflammatory properties of the extracts.

#### 2.4.2. Acetic Acid-Induced Writhing

The writhing method was used to evaluate the analgesic activity of *M. spathulata*. This method is broadly used in analgesic studies due to its sensitivity and capacity to detect molecules with both sides of the brain affected [66,67]. The activity exerted by MS\_L and MS\_B were reported in Table 5. MS\_L and MS\_B showed a strong analgesic effect on acetic acid-induced twisting response during stretching abdominals and in a dose-dependent manner ( $p < 0.001$  with ANOVA, respectively). The maximal inhibition was exerted by both



extracts at the dose of 400 mg/kg (77.21% and 71.32%, respectively for MS\_L and MS\_B), analogous to that of paracetamol at 100 mg/kg (83%,  $p > 0.05$ , respectively) showing the potent analgesic activity of the aerial parts of this species. Acetic acid injection provokes pain by releasing histamine, serotonin, bradykinin, P, and prostaglandins (PGE2a, PGF2a) [68]. These compounds stimulate peripheral nociceptive neurons, increase vascular permeability, and cause pain visceral with the contraction of the abdominal muscles associated with limb elongation and body lengthening [69,70]. The dose-dependent analgesic activity means an increase in the relative content of secondary metabolites in the tested extract, particularly for quinic acid and its derivatives which have been reported for their analgesic activities [59]. The pharmacological mechanism of action could be linked to the synthesis inhibition and liberation of various internal mediators of inflammation and nociceptive, via the increased concentration of the principal compounds found in each extract.

**Table 5.** Effect of MS\_L and MS\_B on nociceptive responses in the acetic acid-induced writhing test.

| Sample      | Dose (mg/kg) | Writhing Number            | Pain Inhibition (%) |
|-------------|--------------|----------------------------|---------------------|
| Vehicle     | -            | 13.60 ± 4.46               | -                   |
| MS_L        | 100          | 8.10 ± 2.33 <sup>a,c</sup> | 40.44               |
|             | 200          | 4.20 ± 2.93 <sup>b</sup>   | 69.12               |
|             | 400          | 3.10 ± 0.96 <sup>b</sup>   | 77.21               |
| MS_B        | 100          | 10.10 ± 1.39 <sup>c</sup>  | 25.74               |
|             | 200          | 6.00 ± 1.17 <sup>a,c</sup> | 55.88               |
|             | 400          | 3.90 ± 0.65 <sup>b</sup>   | 71.32               |
| Paracetamol | 100          | 2.60 ± 1.56 <sup>b</sup>   | 83.03               |

Values were expressed as mean ± s.e.m of the writhing number of five independent experiments. <sup>a</sup>:  $p < 0.05$  vs. vehicle. <sup>b</sup>:  $p < 0.01$  vs. vehicle. <sup>c</sup>:  $p < 0.05$  vs. paracetamol.

### 2.5. Acute Toxicity

In the acute toxicity tests of MS\_L and MS\_B, no evidence of toxicity was observed in mice at doses between 250 and 2000 mg/kg b.w., in skin and fur, respiratory system, food consumption, defecation, and other behaviors, according to OECD [71]. During the three days of monitoring, no unusual behavior or mortality was detected in all treated mice with both extracts. Therefore, the LD<sub>50</sub> values might be higher than the dose of 2000 mg/kg for MS\_L and MS\_B. In accordance with the OECD 423 guideline method [71], MS\_L and MS\_B could be considered as non-toxic supporting the traditional use of *M. spathulata*. The non-toxicity of the plants and their extracts has been confirmed in a number of research studies by OECD protocols [55,72,73].

## 3. Materials and Methods

### 3.1. Plant Material

The bark and leaves of *M. spathulata* were collected in the oriental littoral forest (18°24'27" S, 49°09'20" E, 76 m above sea level) near the Ambodikininina Fokotany in Ampasimadinika Commune of the Toamasina II District, 12 km from the Toamasina City. The voucher specimen was deposited at the Institut Malgache de Recherches Appliquées (IMRA) after the identification performed by Dr Benja Rakotonirina. After the collection, leaves and bark were separated before being dried in a fresh and aerated environment, out of the sunlight and away from the soil. Pharmacological analyses were conducted at IMRA, while phytochemical analyses were performed at the University of Turin (Department of Agricultural, Forestry and Food Sciences—DISAFA) in Italy.

### 3.2. Extracts Preparation

Plant powders consisting of leaves or barks (100 g) were extracted in 250 mL of a mixture of MeOH/water (95/5; v/v) for 24 h at ambient temperature with frequent stirring. The extraction procedure was performed three times. Filtrates were then combined and

dried at 40 °C in a Rotavapor (Büchi R114, Essen, Germany) at reduced pressure allowing leaf methanol extract (MS\_L) and bark methanol extract (MS\_B) of *M. spathulata*.

### 3.3. Animals

The tests were conducted on male and female Swiss albino mice aged between 4 and 5 months with an average weight of  $25 \pm 5$  g. Animals at the IMRA animal house were raised under controlled conditions according to Directive 2010/63/EU of the European Parliament and the Council on the Protection of Animals in Scientific Studies. They were kept in a 12 h dark/12 h light cycle at  $25 \pm 2$  °C and  $65 \pm 5\%$  relative humidity and were fed a standard diet (1420, Livestock Feed Ltd., Port Louis, Mauritius). Prior to the study, the animals fasted overnight. All procedures were approved by the local ethics committee (No. 03/CEA-IMRA/2019).

### 3.4. Analgesic Writhing Test in Mice

The antinociceptive activities of MS\_L and MS\_B were evaluated following the writhing test method defined by Koster et al. [74]. Eight groups of five fasted mice were used:

- Group I: negative control or vehicle where mice were treated orally with normal saline solution (0.9%).
- Group II: positive control where mice were treated orally with paracetamol at a dose of 100 mg/kg body weight (b.w.).
- Groups III–V: mice were treated orally with MS\_L at a dose of 100, 200, and 400 mg/kg b.w., respectively.
- Groups VI–VIII: mice were treated orally with MS\_B at different doses (100, 200, and 400 mg/kg b.w., respectively).

A 100 µL solution of 1.5% acetic acid in 0.9% saline was injected intraperitoneally 1 h after sample administration to induce specific writhing. The writhing number in a period of 5–30 min after the injection of acetic acid was considered. The writhing number of the negative control for each extract was used to calculate the inhibition percentage.

### 3.5. Carrageenan-Induced Mice Paw Oedema Assay

The MS\_L and MS\_B anti-inflammatory properties were performed with slight modifications using the carrageenan-induced mice paw oedema reported by Morris's method [75]. This method consists of inhibiting carrageenan-induced oedema in the posterior leg of mice using a plethysmometer (Ugo Basile 7140, Varese, Italy). Five mice composed each group of fasted animals. In total, eight groups were considered:

- Group I: negative control or vehicle where mice were treated orally with normal saline solution.
- Group II: mice were treated with a dose of 10 mg/kg per indomethacin (positive control).
- Groups III–V: mice were orally treated with MS\_L with a dose of 100, 200, and 400 mg/kg (b.w.), respectively.
- Groups VI–VIII: mice were treated orally with MS\_B at the same doses as groups III–V.

A 100 µL solution of 2% carrageenan in 0.9% saline was injected into the right posterior paw of each mouse 1 h after sample application. Paw volume was measured before the injection and then at 30, 60, 120, and 180 min.

### 3.6. Acute Toxicity Assay

MS\_L and MS\_B oral acute toxicity was assessed following guideline 423 described by the Organization for Economic Co-operation and Development (OECD, 2002) [71]; this method was slightly modified on mice. Fasted animals were divided into nine groups of five:

- Group I: negative control or vehicle where mice were prepared with normal saline solution.
- Group II–V: mice were orally treated with MS\_L with a dose of 250, 500, 1000, and 2000 mg/kg (b.w.), respectively.
- Group VI–IX: mice were orally treated with MS\_B with a dose of 250, 500, 1000, and 2000 mg/kg (b.w.), respectively.

Each toxic effect was monitored for 6 h on the treated animals. These effects on the animals were then evaluated for the next 3 days. Changes in some body functions (e.g., food and water intake, urination, tremors, temperature, respiration, constipation, eye, and skin color), together with behavior, were studied.

### 3.7. Total Polyphenolic Content (TPC)

The Folin–Ciocalteu reagent was employed to evaluate the TPC in the leaves and barks of *M. spathulata* [76]. Five g of leaf or bark powders were macerated for 24 h in the dark with 75 mL of a mixture of methanol–water (95/5; *v/v*) acidified with hydrochloric acid. The mixture was filtered through Whatman filter paper, 185 mm Ø. Ten µL of filtrates were added to 190 µL of extraction solvent, 1000 µL of Folin–Ciocalteu reagent (diluted 1:10), and 800 µL sodium carbonate (7.5%). The last mixture was left in the dark for 30 min, and the absorbance was read using a UV–visible spectrophotometer (1600-PC, VWR International, Radnor, PA, USA) at 760 nm wavelength. Gallic acid standard solution was prepared at 0.02–0.10 mg/mL, according to Donno et al. [77] and the results were reported as gallic acid equivalents (GAE) per 100 g of dry weight (DW).

### 3.8. Anti-Radical DPPH Assay

MS\_L and MS\_B free radical scavenging capacity was measured by using the spectrophotometric radical DPPH method adapted from Sreejayan and Rao [78]. Shortly, 25 µL of the sample methanol solution with a concentration ranging from 2 to 100 µg/mL was added to 175 µL of DPPH methanol solution (0.25 mmol/L) in a 96-well microplate. The mixtures were incubated at room temperature ( $21 \pm 1$  °C) for 30 min. The absorbance was measured at 517 nm with a spectrophotometer Varioskan, (Thermo Fisher Scientific, Waltham, MA, USA). The methanol solution was used as a reference, while the methanol solution of DPPH was employed as a negative control. The antioxidant reference compound used was gallic acid (2.5–40 µg/mL). The results were reported as the mean inhibitory concentration (IC), which was calculated using the equation  $IC(\%) = 100 \times (A_0 - A_1)/A_0$ .  $A_0$  and  $A_1$  represent the absorbance of the negative control and the sample, respectively. The  $IC_{50}$  was determined using a linear regression equation of the inhibition percentage and sample concentration of the extract or gallic acid, and the ordinate was the percentage of scavenging capacity from three replicates.

### 3.9. Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant capacities of the leaves and the barks of *M. spathulata* were evaluated using the FRAP method developed by Benzie and Strain [79]. This procedure is characterized by the ferric reduction ( $Fe^{3+}$ ) to ferrous ions ( $Fe^{2+}$ ) in a solution containing 2,4,6-tripyridyl-S-triazine (TPTZ). Five g of leaf or bark powders were soaked for 24 h in dark with 75 mL of a mixture of methanol–water (95/5; *v/v*) acidified with HCL. The mixture was filtered through Whatman filter paper, 185 mm Ø. Ten µL of filtrates were added to 20 µL of extraction solvent, 900 µL of FRAP reagent (0.3 M acetate buffer pH 3.6/TPTZ 10 mM in 40 mM HCl/20 mM ferric chloride; 84/8/8; *v/v/v*), 90 µL of pure water. Finally, it was placed at 37 °C in a thermostatic bath for 30 min. Blank was prepared by replacing the sample with the extraction solvent. The absorbance was quantified at a wavelength of 595 nm by using a UV–vis spectrophotometer (model 1600—PC, VWR International, Radnor, PA, USA) [80].  $FeSO_4 \cdot 7H_2O$  standard solution was prepared at 100–1000 µmol/mL, and the results were expressed in millimoles of iron ions ( $Fe^{2+}$ ) per kilogram of dried weight.

### 3.10. HPLC Fingerprint Analysis

#### 3.10.1. Sample Preparation

Leaf or bark powders (5 g) were extracted in 75 mL of a mixture of methanol–water (95/5; *v/v*) with HCL for 24 h in the dark. The mixture was filtered through Whatman filter paper (185 mm Ø), and the filtrates were recovered and re-filtered with a GHP filter (45 µm) and kept at 4 °C until analysis.

#### 3.10.2. Chromatographic Analysis

The chromatographic analysis of leaf and bark extracts of *M. spathulata* was carried out using an Agilent 1100 HPLC system (Santa Clara, CA, USA) equipped with a G1311A quaternary pump, a manual injection valve and a 20 µL sample loop coupled to an Agilent G1315D diode array UV/Vis detector. Twenty µL of sample were injected, and the chemical components of samples were separated through a C18 column Phenomenex Kinetex, while an NH2 column SphereClone was used only for sugar analysis [80]. A total of five chromatographic methods described in the Supplementary Materials were used to quantify the polyphenols, the organic acids, the vitamins, and the sugars. The detection of bioactive components in samples was based on the comparison of their UV spectra and retention times to the ones of authentic standards using the same experimental conditions. Calibration curves were established by tracing the peak area (*y*) against the concentration (*x*) of each standard. The linearity was then measured by the values of the correlation coefficient ( $R^2$ ) [81]. The result of any sample is the mean of three repetitions, expressed as mg/100 g of DW, except for organic acids and sugars (g/g DW).

### 3.11. Statistical Analysis

All samples were set up and analyzed at least in triplicate. The data were expressed as mean values  $\pm$  standard deviations or standard error mean values. Results were statistically evaluated using the student's *t*-test and ANOVA test for comparison of means (SPSS 22.0 software), followed by Tukey's HSD multiple interval test. Values of  $p < 0.05$  are considered as statistically significant. Pearson's coefficient (*R*) was utilized to investigate the correlations between phytochemicals, TPC, and antioxidant activity. All positive correlations found were then compared and confirmed using the bioinformatics approach outlined by Gfeller et al. [82], which was used to predict potential targets and pharmacokinetics. To identify the putative targets of key compounds, all chemical structures were prepared and converted into SMILES (Simplified Molecular Input Line Entry System) using ACD/ChemSketch (version 12.0). The compound SMILES were then put into the Swiss Target Prediction network (<http://www.swisstargetprediction.ch/>, accessed on 8 April 2024), and the resulting targets were obtained.

## 4. Conclusions

This study represents the first investigation of the phytochemical and pharmacological properties of *Morella spathulata*, confirming its importance in traditional Malagasy medicine. Extracts of leaves and bark have demonstrated high contents of total phenolic content compared with other Malagasy species, and bioactive compounds, as well as antioxidant, anti-inflammatory, and analgesic properties due to phenols and organic acids, most notably quinic acid, and benzoic and cinnamic acids, such as ellagic and ferulic acids. The synergy of these molecules, expressed in their positive correlation, could positively influence health. Furthermore, the Swiss Target Prediction platform revealed interesting interactions, indicating that ferulic acid targets carbonic anhydrase (CA2), which plays a crucial role in pH regulation and has implications for anti-inflammatory effects. Instead, ellagic acid was found to interact with G protein-coupled receptor 35 (GPR35), suggesting possible involvement in immune regulation and modulation of inflammation. These results provide preliminary information on the molecular strategies driving the therapeutic effects of the plant and highlight its multiple pharmacological properties. By understanding the molecular targets of bioactive compounds, it may be possible to guide targeted therapies

and preventive strategies. However, it is crucial to recognize the conservation challenges that this species faces due to illegal logging and habitat loss. Therefore, simultaneous conservation efforts are needed to study effective propagation and domestication techniques, involve local communities in conservation through educational programs, implement selective harvesting measures, establish protected areas that can mitigate over-exploitation and ensure the long-term availability of *M. spathulata*. Indeed, biotechnological solutions, such as in vitro micropropagation, cell suspension, or callus cultures could offer the possibility of producing plant material on a large scale or extracting bioactive compounds avoiding the use or in some cases the abuse of natural resources. These efforts emphasize a dual commitment to medicinal plant exploration and environmental conservation. Considering the scarcity of research on this species, this study may help to reduce the gap in the understanding of its therapeutic potential and provide the basis for future studies.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants13202899/s1>, Table S1: Chromatographic conditions of each used method.

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## Article

# Pharmacological Potential and Electrochemical Characteristics of *Typha angustifolia* Pollen

Janielle Mari S. Abadilla <sup>1,2,†</sup>, Bor-Yann Chen <sup>3,†</sup>, Mike Anthony D. Ganzon <sup>1</sup>, Alvin R. Caparanga <sup>1,2</sup>, Kristopher Ray S. Pamintuan <sup>1</sup>, Lemmuel L. Tayo <sup>1,4</sup>, Chung-Chuan Hsueh <sup>3</sup>, Cheng-Yang Hsieh <sup>3,5</sup>, Ling-Ling Yang <sup>6,7</sup> and Po-Wei Tsai <sup>5,\*</sup>

<sup>1</sup> School of Chemical, Biological, and Materials Engineering and Sciences, Mapúa University, Manila 1002, Philippines; jmsabadilla@mymail.mapua.edu.ph (J.M.S.A.); madganzon@mymail.mapua.edu.ph (M.A.D.G.); arcaparanga@mapua.edu.ph (A.R.C.); krspamintuan@mapua.edu.ph (K.R.S.P.); lltayo@mapua.edu.ph (L.L.T.)

<sup>2</sup> School of Graduate Studies, Mapúa University, Manila 1002, Philippines

<sup>3</sup> Department of Chemical and Materials Engineering, National I-Lan University, Yilan 260, Taiwan; boryannchen@yahoo.com.tw (B.-Y.C.); cchsueh@niu.edu.tw (C.-C.H.); d339108001@tmu.edu.tw (C.-Y.H.)

<sup>4</sup> Department of Biology, School of Health Sciences, Mapúa University, Makati 1200, Philippines

<sup>5</sup> Department of Food Science, National Taiwan Ocean University, Keelung 202, Taiwan

<sup>6</sup> School of Pharmacy, College of Pharmacy, Taipei Medical University, Taipei 110, Taiwan; llyang@tmu.edu.tw

<sup>7</sup> Department of Acupuncture, American College of Acupuncture & Oriental Medicine, Houston, TX 77063, USA

\* Correspondence: powei@mail.ntou.edu.tw

† These authors contributed equally to this work.

**Abstract:** *Typha angustifolia* L. (TA) pollen has been utilized as a traditional Chinese medicine for treating various internal and external traumas. Moreover, bioactive compounds possess diverse pharmacological activities. This study aims to evaluate the antiviral properties of TA based on its ability to generate bioenergy, capable of inhibiting viruses. TA pollens were extracted using water and ethanol solvents. These extracts were utilized to identify the phytochemical contents and correlate with the antioxidant activity via 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. HPLC analysis was conducted to identify its electron-shuttling compositions. The bioenergy-generating characteristics were determined via microbial fuel cells. The water extract (TA-W) showed higher antioxidant activity due to a higher phenolic and flavonoid content compared to the ethanol extract (TA-E). Quercetin-3-O-(2<sup>G</sup>-α-L-rhamnosyl)-rutinoside, quercetin-3-O-neohesperidoside, and quercetin are the electron shuttles (ES) identified out of the 11 compounds. TA obtained a  $1.39 \pm 0.10$  amplification factor of power generation that indicates potential bioenergy-generating and associated antiviral characteristic properties. The findings may provide a foundation for developing antiviral medications specifically designed to target virus-related diseases, while minimizing the risk of drug toxicity and reducing the costs of drug development.

**Keywords:** antiviral; electron shuttles; flavonoids; microbial fuel cells; *Typha angustifolia*

## 1. Introduction

For more than 2000 years, traditional Chinese medicine (TCM) has been utilized in the healthcare system for various treatment protocols, diagnostic assessments, and illness prevention. Several applications of TCM include herbal medicine, acupuncture, moxibustion, and other non-medication therapies. Despite its extensive application, debates still rise when TCM and Western medicine are compared based on its treating efficacy. Secondary metabolites found in herbal medicines exhibit various therapeutic effects such as antioxidant, anti-inflammatory, and immunomodulatory properties. Recently, TCM has been reported to contain compounds with electron shuttle (ES)-like structures (e.g., phenolics, anthraquinone, and flavonoid) [1,2]. ESs were specifically classified from the category of antioxidants that were misunderstood in the academic field due to redox-mediating electrons



freely resonating within the chemical structure (e.g., the aromatic rings and flavonoids). Their sustainable potential capabilities to mediate resonant electrons within the ESs were not like electrons, that could be consumed for excitation as antioxidants. As recent findings exhibited, these structures are investigated as prospective candidates for the selection of antiviral properties [3]. Hence, this discovery for new effective antiviral medications can aid to bridge the consensus gap between Western medicines and TCM for a wide range of applications.

*Typha angustifolia* L. (TA), commonly referred to as narrow-leaved cattail, is a perennial aquatic herb. It is one of the 30 species classified under the genus *Typha*, which belongs to the *Typhaceae* family [4]. This plant is commonly found in Central Asia, Northern Africa, and North America, specifically in riverbanks, wetlands, and lake shores of tropical regions, growing up to three to seven meters in height [5]. The phytoremediation properties of TA are due to its capacity to store and tolerate high concentrations of various metals [6,7]. Conversely, one of the highly used TCMs to consider is the dried pollen of TA. It has a bright yellow or green appearance, with a diameter that ranges between 19 and 27  $\mu\text{m}$  [8]. TA pollen has been recognized in managing external wounds (e.g., abscesses) and internal bleeding (e.g., dysmenorrhea and dysentery) [9]. Additionally, it promotes urination, relieving conditions like urinary bleeding and strangulation [10]. In traditional medicine, it has been used to treat leprosy [9,11], hyperlipidemia, and to improve microcirculation in China [12]. Beyond its folkloric uses, in vivo studies have demonstrated the anti-inflammatory activity of TA extract in carrageenan-induced rats, showing promising results for treating both acute and chronic conditions [9]. Other studies have further highlighted its pharmacological activities, including immunosuppressive, wound healing, and anti-atherosclerotic effects [13–16]. Aside from its medicinal properties, TA pollen is used in cooking as a thickener or flour substitute [17]. Despite the numerous investigations on TA pollen, the scarcity of knowledge on compounds containing ES-like structures is still evident. Furthermore, rare research explored the antiviral properties of TA pollen for developing new drugs, utilizing electrochemical analysis through microbial fuel cells (MFCs).

Hsueh et al. [18] demonstrated the bioenergy-generating capabilities of ES compounds with the use of MFC as the platform for electrochemical evaluation. ES (or redox mediators) are functioned to improve the efficiencies of an electron transfer and bioenergy generation to mediate the electron transport chain in cells. Polyphenols derived from natural resources are suitable candidates for ESs. This is due to their low biotoxicity potency, high biocompatibility, and reversible attribute for power-generating capabilities. However, because of the weak acidic nature of polyphenols, it is essential to account for various factors to ensure favorable conditions are observed, including pH levels, the redox potential, and water solubility [18]. Hence, not all plant species that have an abundance of antioxidants which would be directly considered to be ES-plentiful. Furthermore, it was found that herbal extracts containing *o*-diOH and flavonoid compounds play both crucial antioxidant and ES roles which exhibit antiviral activity.

Viral infection symptoms vary from mild to severe, which depends on the immune response and the viral load in the host cells. One of the leading viral diseases in the world is caused by the family Flaviviridae. These are a single-stranded, positive, and encapsulated RNA virus. Dengue virus (DENV), hepatitis C virus (HCV), and Japanese encephalitis virus (JEV) are the only few viruses that belong to Flaviviridae. Both DENV and JEV belong to the genus *Flavivirus* while HCV belongs to the genus *Hepacivirus*. Dengue, a vector-borne disease caused by DENV, is transmitted to humans by *Aedes* mosquitoes. Common symptoms may range from a mild flu-like condition to hypovolemic shock [19]. Japanese encephalitis is transmitted by *Culex* mosquitoes carrying JEV and is prevalent among individuals residing in rural Asian regions. This disease infects nearby tissues and lymph nodes upon transmission. Common symptoms include abdominal pain and anorexia [20,21]. Conversely, hepatitis C is a bloodborne disease that causes progressive liver damage, culminating in liver cirrhosis and hepatocellular carcinoma. Clinical indications of hepatitis C are fatigue, vomiting, and abdominal pain [22–24]. Even though such



symptoms mentioned are often typical and can sometimes be overlooked, these diseases are notorious for causing catastrophic infections, leading to high morbidity and mortality rates over the years.

Due to the concerning statistics and life-threatening effects of diverse viral diseases, a critical focus on exploring viable medications is being worked on as a priority to preserve lives worldwide. Recent progress in understanding the electrochemical behavior of herbal medicines has unveiled their potential to possess antiviral properties. Hence, this study aims to identify the bioenergy-generating and ES characteristics of TA pollen. Furthermore, this study aims to identify the bioenergy-generating and electrochemistry-associated characteristics of TA pollen and utilize its electrochemical behavior as a preliminary screening technique to determine its antiviral capability.

## 2. Materials and Methods

### 2.1. Sample Preparation and Extraction

The dried TA pollens were purchased from a local TCM store in Tainan, Taiwan, and authenticated by Dr. Ling-Ling Yang from Taipei Medical University. A voucher specimen (#NIU-TA-001) was deposited at the Department of Chemical and Material Engineering in National I-Lan University. The extraction process followed a 1:20 (g/mL) sample-to-solvent ratio, using 100 g of TA pollen with 2 L of distilled deionized (DD) water or ethanol. The water sample was boiled in a traditional Chinese decoction pot until reduced to 200 mL, while the ethanol sample was extracted using reflux for two hours at 60 °C. Solids were removed by vacuum filtration, and the filtrate was concentrated using a rotary evaporator to obtain crude extracts. The crude extracts were then freeze-dried to achieve a powder-like consistency.

### 2.2. Phytochemical Analysis

To further evaluate electrochemical characteristics, total polyphenols (TP), flavonoid (TF), and condensed tannin (TCT) content were quantitatively determined with the use of water extracts (TA-W) and ethanol extracts (TA-E).

#### 2.2.1. Total Phenolic Content (TPC) Analysis

A gallic acid-based standard solution (1 mg/mL) and sample solution (10 mg/mL) were used to determine TPC of TA pollen with minor modifications from Tsai et al. [3]. The solution was serially diluted by two-fold to obtain seven concentrations (1000 to 15.6 µg/mL). Then, 20 µL of each solution was combined with 100 µL of 0.2 N Folin–Ciocâlteu reagent and 80 µL of 7.5% Na<sub>2</sub>CO<sub>3</sub>. After a 30 min reaction, 200 µL aliquots were placed in a 96-well plate in triplicates for accuracy. The absorbance was measured at 600 nm using an ELISA microplate reader (Multiskan SkyHigh, Thermo Scientific, Waltham, MA, USA).

#### 2.2.2. Total Flavonoid Content (TFC) Analysis

A rutin-based standard solution (1 mg/mL) and sample solution (10 mg/mL) were used to determine the TFC in TA pollen, following a modified version of Tsai et al. [3]. Each 500 µL standard solution was reacted with 500 µL of 2% AlCl<sub>3</sub> for 15 min. Then, 200 µL aliquots were transferred in 96-well plate. Then, the absorbance of the mixture was measured at 430 nm.

#### 2.2.3. Total Condensed Tannin (TCTC) Analysis

A catechin-based standard solution (1 mg/mL) and sample solution (10 mg/mL) were used to determine the TCTC of TA pollen, following the method of Tsai et al. [3]. For this, 300 µL of solution was reacted with 600 µL of vanillin in 80% H<sub>2</sub>SO<sub>4</sub> and incubated in a water bath at 50 °C for 15 min. Then, 200 µL aliquots were transferred in a 96-well plate and the absorbance was measured at 530 nm.

### 2.3. Antioxidant Activity

Antioxidant activity of TA samples was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric-reducing antioxidant power (FRAP) assays.

#### 2.3.1. DPPH Free Radical Scavenging Activity

A 200  $\mu$ M DPPH solution was prepared as the reaction reagent. A standard solution (1 mg/mL) was made by dissolving 10 mg of ascorbic acid in 10 mL of ethanol [3]. It was followed by two-fold serial dilutions to achieve concentrations from 1000  $\mu$ g/mL to 7.8  $\mu$ g/mL. Then, 50  $\mu$ L of each sample (10 mg/mL) and standard solution was reacted with 150  $\mu$ L of DPPH solution. The absorbance was measured at 517 nm. The radical scavenging activity (%RSA) was calculated using Equation (1), and linear regression was applied to estimate the half maximal inhibitory concentration (IC<sub>50</sub>) at 50% RSA.

$$\%RSA = \left[ 1 - \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \right] \times 100 \quad (1)$$

%RSA = % Radical Scavenging Activity

A<sub>sample</sub> = absorbance of sample

A<sub>control</sub> = absorbance of control

A<sub>blank</sub> = absorbance of blank

#### 2.3.2. Ferric Reduction Antioxidant Power (FRAP) Assay

A FRAP reagent was prepared using a 10:1:1 ratio of pH 3.9 acetate buffer, 5 mM 2,4,6-Tripyridyl-S-triazine (TPTZ) solution, and 20 mM FeCl<sub>3</sub>·6H<sub>2</sub>O solution, following a method from Tsai et al. [3]. For this, 2 mg/mL Trolox stock solution was made by dissolving 10 mg of Trolox in 5 mL of a 2:3 ethanol-DD water mixture. Two-fold serial dilutions were performed to obtain concentrations from 2000  $\mu$ g/mL to 31.3  $\mu$ g/mL. For the reaction mixture, 50  $\mu$ L of each solution was mixed with 1450  $\mu$ L of FRAP reagent. Then, 200  $\mu$ L aliquots were transferred to a 96-well plate. The absorbance was measured at 593 nm.

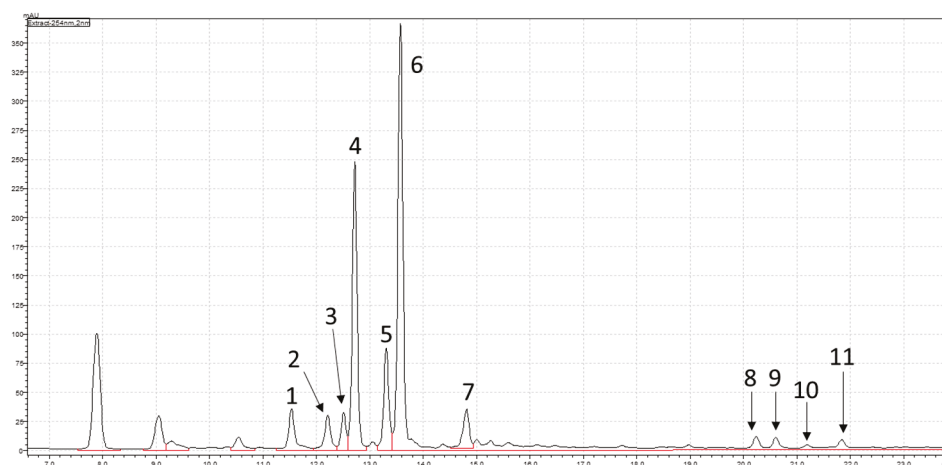
### 2.4. High-Performance Liquid Chromatography (HPLC) Analysis

In conducting the high-performance liquid chromatography (HPLC) analysis, Hyperasil™ BDS C18 column (250 × 4.6 mm, 5  $\mu$ m, Thermo Fischer Scientific Inc.), a 10 mg/mL ethanol extract, was prepared. It was filtered using a 0.45  $\mu$ m syringe filter, with 1 mL transferred into each vial. A Shimadzu HPLC model LC-2050C 3D was used to separate and identify the major compounds in TA pollen. The chromatographic analysis utilized Mobile Phases A (water), B (acetonitrile), and C/D (methanol). The column oven was maintained at 40 °C with a flow rate of 1 mL/min. The gradient program started at 0 min with 95% Mobile Phase A and 5% Mobile Phase B, reaching 100% Mobile Phase B at 60 min [3].

HPLC analysis identified the bioactive compounds present in TA-E. The peaks from the chromatographic fingerprint (Figure 1) were analyzed and compared to reference compounds from Tao et al. [25] for validation. The analysis identified two major compounds, isorhamnetin-3-O-neohesperidoside (I3ON) and typhaneoside, based on their peak intensity, area, and retention time. These compounds were also confirmed as the primary flavonoids in TA pollen, as reported in the previous literature [26].

Figure 1 shows the peaks displayed on the chromatographic fingerprint of TA-E from 2D HPLC-PDA. The following compounds detected include: (1) quercetin-3-O-(2<sup>G</sup>- $\alpha$ -L-rhamnosyl)-rutinoside; (2) quercetin-3-O-neohesperidoside; (3) kaempferol-3-O-(2<sup>G</sup>- $\alpha$ -L-rhamnosyl)-rutinoside; (4) isorhamnetin-3-O-(2<sup>G</sup>- $\alpha$ -L-rhamnosyl)-rutinoside (typhaneoside); (5) kaempferol-3-O-neohesperidoside; (6) isorhamnetin-3-O-neohesperidoside;

(7) isorhamnetin-3-*O*-rutinoside; (8) quercetin; (9) naringenin; (10) kaempferol; and (11) isorhamnetin.



**Figure 1.** Chromatographic fingerprint of EtOH extract of TA pollen from 2D HPLC-PDA at 245 nm.

## 2.5. Power-Density Determination via MFCs

### Microbial Fuel Cell Framework and Microbial Cultures

An H-type double chamber (DC) MFC was used to assess the bioenergy generation of TA pollen. Graphite anode and cathode electrodes (Grade: IGS743; Central Carbon Co., Ltd., Taipei, Taiwan) were soaked in electrolyte and culture broth, with an area of 1.649 cm<sup>2</sup>. A 200 mL operating volume was isolated by a proton exchange membrane (DuPont™ Nafion® NR-212, Wilmington, DE, USA) with an immersed area of 0.000452 m<sup>2</sup> in each chamber. In the cathodic chamber, the 6.38 g K<sub>3</sub>Fe(CN)<sub>6</sub> and 17.42 g K<sub>2</sub>HPO<sub>4</sub> electrolyte was dissolved in 200 mL deionized water. The anodic chamber contained a culture broth prepared with *Aeromonas hydrophila* (National Center for Biotechnology Information or NCBI ID = 644), a Gram-negative, electroactive bacterium that was selected for its ability to degrade organic compounds and form biofilm on electrodes, enhancing electron transfer [27]. The bacteria were precultured in 100 mL LB broth (Difco™ LB Broth, Miller; Luria-Bertani, Minato, Japan) at 30 °C and 125 rpm for 12 h. Then, 1 mL of the precultured broth was added to 18 flasks, each with 100 mL sterilized LB broth, cultured until optical density reached 2.1 at 600 nm. The anodic chamber was supplied with 200 mL of culture broth, and a 1.2 g of TA sample was dissolved in 12 mL of deionized water and ethanol. Power density was measured with TA concentrations of 500 mg/L, 1000 mg/L, and 2000 mg/L. The chamber and electrode were rinsed with sterile deionized water after each concentration. The optical density was maintained at 2.1, with Blank 1 used before the 500 mg/L sample and Blank 2 after the 2000 mg/L sample for consistency. Lastly, 0.1 mL of 0.6 M dopamine was used as a standard after Blank 2.

## 2.6. Power Density

The power generation of MFCs supplemented with TA extracts was tested using a D/A system (DAS 5020; Jiehan Tech Corp., Taichung City, Taiwan) to measure voltage and electric current over time. For consistency, the external resistance of the MFC was set to 1 kΩ [3]. Power and current densities were calculated using standard equations:

$$P_{\text{density}} = \frac{V_{\text{MFC}} \times I_{\text{MFC}}}{A_{\text{anode}}} \quad (2)$$

$$I_{\text{density}} = \frac{I_{\text{MFC}}}{A_{\text{anode}}} \quad (3)$$

Thermo The working area of the graphite anode,  $A_{\text{anode}}$ , was used for calculations. The voltage of the highest power point ( $V_{\text{MFC}}$ ) and current of the highest power point

( $I_{MFC}$ ) were identified through Linear Sweep Voltammetry (LSV) (Jiehan 5600, Jiehan Technology Corp., Taichung City, Taiwan). The LSV plot was used to determine  $V_{MFC}$  by locating the highest current–voltage product, followed by identifying  $I_{MFC}$  at the same point.

### 2.7. Statistical Analysis

In the phytochemical analysis (Section 3.1) and antioxidant activity (Section 3.2), the tabulated results were displayed as average  $\pm$  standard deviation from the three replicates. Analysis of Variance (ANOVA) was used to analyze the readings, employing a significance level of  $p < 0.05$  using Microsoft Excel® v16.0 software. Descriptive statistics were used to assess the variability of the parameters.

## 3. Results and Discussion

### 3.1. Total Phytochemical Analysis

Table 1 summarizes all the data obtained from the phytochemical analysis. With a determination coefficient ( $R^2$ ) of more than 0.90, the calibration curves explicitly demonstrated a promising linear relationship between the signal and the concentration of the reference standards. This implies that all the polyphenols, flavonoids, and condensed tannins found in the samples were within QA/QC ranges. TP generated a standard curve with an equation of  $y = 5.367x - 0.0014$  ( $R^2 = 0.9992$ ) expressed in gallic acid equivalents (GE). TF obtained a linear equation of  $y = 27.533x + 0.0459$  ( $R^2 = 0.9999$ ) expressed in rutin equivalents (RE). TCT generated the linear equation  $y = 7.2500x + 0.3034$  ( $R^2 = 0.9944$ ) expressed in catechin equivalents (CaE). TA-W had a higher TP and TF content than TA-E, obtaining  $32.6019 \pm 0.0404$  mg/g and  $31.4541 \pm 0.0208$  mg/g, respectively. Conversely, the TCT content of TA-E was higher with a value of  $44.2746 \pm 0.1122$  mg/g (ca. 2.79 folds to TA-W). This suggests that water is a somewhat more favorable solvent in extracting the phenolic and flavonoid content. In contrast, an ethanol solvent is preferably utilized in achieving an optimal amount of condensed tannin extract.

**Table 1.** Total phytochemical content of *T. angustifolia* extracts.

| Sample            | Total Phenolic Content<br>(mg GAE per g CE) | Total Flavonoid Content<br>(mg RE per g CE)  | Total Condensed<br>Tannin Content<br>(mg CE per g CaE) |
|-------------------|---------------------------------------------|----------------------------------------------|--------------------------------------------------------|
| TA-W              | $32.6019 \pm 0.0404$                        | $31.4541 \pm 0.0208$                         | $15.8784 \pm 0.0361$                                   |
| TA-E              | $24.4638 \pm 0.0289$                        | $27.3874 \pm 0.0361$                         | $44.2746 \pm 0.1122$                                   |
| Standard<br>curve | $y = 5.367x - 0.0014$<br>( $R^2 = 0.9992$ ) | $y = 27.533x + 0.0459$<br>( $R^2 = 0.9999$ ) | $y = 7.2500x + 0.3034$<br>( $R^2 = 0.9944$ )           |

GAE, gallic acid equivalent; RE, rutin equivalent; CaE, catechin equivalent; CE, crude extract; TA-W, *T. angustifolia* water extract; TA-E, *T. angustifolia* ethanol extract.

Phytochemicals play a significant role in the health advantages of plant-based diets due to their pharmacological properties. They are a class of diverse compounds that fall into several groups such as phytosterols, polyphenols, flavonoids, carotenoids, and organosulfur compounds [28]. Phenolic compounds are recognized to possess antioxidant properties. Its redox potential assists to function as chelating agents of metal ions or hydrogen donors. High stability is conferred based on the radical produced during the radical neutralization process by an *ortho* configuration of hydroxyl groups. Additionally, greater antioxidant contents are reflected in a higher amount of hydroxyl groups [29,30]. Hsueh et al. [18] discussed that polyphenols are found to act as an ES where it can be reversibly expressed for the bioenergy-generating capacity under favorable conditions. In addition, it is essential to consider various factors (e.g., concentration, pH level, solubility, and biotoxicity) as they can affect the reduction potential of polyphenol compounds.

The chemical structure of polyphenols can be categorized based on the presence of several phenolic groups, classified as flavonoids and non-flavonoids. Plants are omnipresent sources of flavonoids, a broad class of polyphenolic chemicals with a benzoyl- $\gamma$ -pyrone

structure [31]. Aside from this, a flavonoid's bioactivity is determined by its structural substitution patterns in its C6-C3-C6 rings, which serves as the basic skeleton [32]. Flavonoids are abundant in human diets and are recognized in combating various viral infections through multiple stages such as viral entry, replication, and protein translation [33]. On the other hand, condensed tannins are polymers that are developed from two to more leucoanthocyan flavan-3,4-diols [34].

Numerous plant-based polyphenols have been identified and are becoming more significant in the modification of antiviral drugs due to their inexpensive production, accessibility, and minimal side-effects. In addition, negative impacts from viruses are not limited to its replication, rather how the immune system responds to infections, including oxidative stress and inflammation. Thus, combining both antioxidant and antiviral medication could exhibit more potent effects for treating viral infections and counteracting the emergence of antiviral resistance [35,36]. Based on the results obtained, the phenolic, flavonoid, and condensed tannin contents reflect on the prospective health benefits that are found in TA. This only applies to how TA can be a prospective candidate for the in-depth assessment and clinical research of drug development on various viral diseases such as dengue fever, hepatitis C, and Japanese encephalitis. Also, the results show that using different solvents can vary the optimal amount of phytochemical content extraction.

### 3.2. Antioxidant Activity Assessment

The antioxidant activity was reported to be correlated to many disease-resisting capabilities. Thus, revealing such activities was of great importance in herbal medication. As shown in Table 2, the standard curve generated in doing the DPPH scavenging activity is  $y = 2731.35x - 4.3973$  with a determination coefficient ( $R^2$ ) of 0.9981, expressed as an ascorbic acid equivalent. Ascorbic acid is used as a positive control in the said assessment, with an  $IC_{50}$  value of  $0.0199 \pm 0.0004$  mg/mL. The FRAP assay generated a standard curve with a linear equation of  $y = 2.2024x + 0.1548$  with  $R^2 = 0.9919$ , as expressed in the Trolox equivalent. Comparing the values obtained from the crude extracts, TA-W has a lower  $IC_{50}$  value ( $0.8784 \pm 0.0117$ ) and higher FRAP assay value ( $35.2568 \pm 0.0145$ ). On the other hand, TA-E obtained an  $IC_{50}$  value and FRAP assay value of  $2.4073 \pm 0.1091$  and  $25.9639 \pm 0.0167$ , respectively. Both TA-W and TA-E were higher compared to ascorbic acid. However, TA-E was obtained to have a greater difference with ascorbic acid.

**Table 2.** Antioxidant assessment of *T. angustifolia* water and ethanol extracts.

| Samples        | DPPH $IC_{50}$ (mg per mL)                    | FRAP (mg TE per g CE)                        |
|----------------|-----------------------------------------------|----------------------------------------------|
| TA-W           | $0.8784 \pm 0.0117$                           | $35.2568 \pm 0.0145$                         |
| TA-E           | $2.4073 \pm 0.1091$                           | $25.9639 \pm 0.0167$                         |
| Ascorbic acid  | $0.0199 \pm 0.0004$                           | -                                            |
| Standard curve | $y = 2731.35x - 4.3973$<br>( $R^2 = 0.9981$ ) | $y = 2.2024x + 0.1548$<br>( $R^2 = 0.9919$ ) |

TE, Trolox equivalent; CE, crude extract; TA-W, *T. angustifolia* water extract; TA-E, *T. angustifolia* ethanol extract.

Antioxidants found in plants have received considerable attention because of their ability to prevent damage caused by oxidative stress. Oxidative stress happens when there is an increase in the reactive oxygen species (ROS) level from ROS formation and detoxification. This process will then lead to a disturbed cellular function that causes the development of life-threatening diseases [37,38]. Generally, antioxidants follow two mechanisms in scavenging free radicals: by donating hydrogen to generate stable compounds, and by reducing any compounds (i.e., metals, carbonyls, free radicals) by transferring an electron [38]. In this study, the DPPH radical scavenging assay and FRAP assay were conducted as in vitro analyses to quantitatively determine the antioxidant properties of TA pollen. The  $IC_{50}$  value obtained in the experiment dictates the concentration of antioxidants needed to decrease the initial DPPH concentration by 50%. The solution has a purple hue and develops into a colorless or yellow color when neutralized by radical scavengers.



Hence, the lower IC<sub>50</sub> interprets greater antioxidant activity. In contrast, the FRAP assay measures the capacity of antioxidants in reducing ferric ions under acidic conditions. Antioxidants are assessed based on their capability to reduce Fe(III)/tripyridyltriazine complexes. This requires a higher FRAP assay value which indicates a higher level of antioxidant activity. Between the DPPH free radical scavenging activity of TA-W and TA-E, it reveals that water is a more appropriate solvent in achieving the maximum antioxidant activity from TA pollen. Based on its phytochemical analysis, phenolic and flavonoid compounds made a significant contribution to the antioxidant activity of TA-W. This is because TA-W has a lower IC<sub>50</sub> value and a higher phenolic and flavonoid content.

### 3.3. HPLC Analysis of *T. angustifolia* Extract

To exhibit the efficacy of medicinal herbs, showing the presence of such effective compositions is of great significance via HPLC analysis.

The diverse pharmacological effects (e.g., the synergistic interaction of disease treatment) attributed to TCM stem from their bioactive components like phenols, flavonoids, tannins, and phenolic acids. This section provides several therapeutic effects of the bioactive compounds detected in the TA extract that are also present in other herbal and dietary plants. For example, quercetin and kaempferol are under the subgroup flavonols. Flavonols are commonly found in significant quantities in plant sources, typically in glycoside forms. They derive from 3-hydroxyflavone backbone, and are often linked with a ketone group [39,40]. Apart from their antioxidant properties, prior research indicated that both compounds have the potential to relieve symptoms associated with Alzheimer's disease (AD). Furthermore, they suggested that a combination of herbal extracts (e.g., *Camellia sinensis* L., *Cyperus rotundus* L., and *Ginkgo biloba* L.) abundant in quercetin and kaempferol could address AD-related brain disorders [41]. Both compounds have also emerged with a potential to possess antihypertensive, anti-atherosclerotic, and cardioprotective effects which makes this a potential treatment for cardiovascular disease [42,43]. Stemming from the same subgroup, isorhamnetin originates as a quercetin molecule that has undergone methylation. Isorhamnetin extracted from *Hippophae rhamnoides* L. showed potent anti-tumor activity. Moreover, other research suggested that it promotes the carbohydrate metabolism and exhibits anti-diabetic activities [44,45]. Another constituent found within the TA extract is kaempferol-3-O-neohesperidoside, categorized as a flavonoid glycoside and belonging to the family of flavonoid derivatives. The said compound is also present in *Dyopsis pambana* which exhibits the highest antidiabetic activity that inhibits alpha glucosidase enzymes in vitro and in silico [46]. On the other hand, *Phoenix dactylifera* L. extracts contain substances such as phenolics and flavonoids (flavanones, isoflavanones, and fava-3-ols) with antibacterial and antiviral properties, which was found with the use of GC-MS analysis [47]. In fact, polyphenolics have also been used in the food industry as they possess antimicrobial activity. *Punica granatum* peel extracts were found to contain flavonoids that acted as reducing and capping agents [48]. Other derivatives of isorhamnetin that were also detected are isorhamnetin-3-O-rutinoside and isorhamnetin-3-O-neohesperidoside. These compounds own the antioxidant and wound healing properties of the dried flowers of *Calendula officinalis* L. [49,50]. Furthermore, naringenin, categorized within the flavanone subgroup, is present in TA pollen. Flavanones, aromatic and colorless ketones derived from flavones, are commonly distributed in various *Citrus* species (e.g., lemon, orange, and clementine). Furthermore, they are known to possess diverse biological activities such as antitumor, antiviral, antibacterial, and anti-inflammatory effects [51,52]. Meanwhile, typhaneoside from *Typha orientalis* L. was also reported to alleviate the symptoms of non-alcoholic fatty liver disease (NAFLD) due to its resistance to glucose and lipid metabolism disorders [53].

As shown, all identified bioactive compounds were polyphenols, specifically defined as flavonoids. It was mentioned earlier that polyphenols obtained from natural resources were bioenergy-generating and electron-medicating. This is due to the chemical characteristics (i.e., aromaticity, nature of substituent, and its positions) found in organic ESs that are also inherent in polyphenols [18]. The chemical configuration of an ES significantly

impacts the stability and dispersion of electrons and radicals, which is crucial for bioenergy extraction.

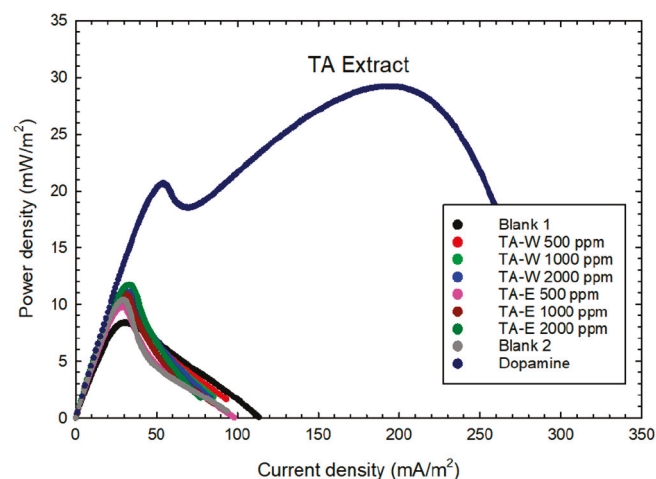
### 3.4. Power Density Measurements

In fact, such electron-transferring potential of test herbal extracts should be directly reflected upon bioelectronic-generating characteristics via MFC platforms. Table 3 presents the power-generating performance and its corresponding amplification factor. It shows that the samples were assessed using varying concentrations (i.e., 500 mg/L, 1000 mg/L, and 2000 mg/L). The result reveals that TA-E with 2000 mg/L obtained the highest power density ( $11.71 \pm 0.34 \text{ mW/m}^2$ ) and amplification factor ( $1.39 \pm 0.10$ ) among all the samples. As shown in Figure 2, the top rank power density of pure chemical dopamine was adopted as a reference standard and control in the system. It is anticipated for chemical mixture-bearing herbal extracts to obtain lower values of the power density.

**Table 3.** Power density analysis and amplification factor of *T. angustifolia* water and ethanol extracts at different concentrations. Amplification factor with respect to 1.

| Samples        | Power Density ( $\text{mW/m}^2$ ) | Amplification Factor |
|----------------|-----------------------------------|----------------------|
| Blank 1        | $8.39 \pm 0.36$                   | 1                    |
| TA-W 500 mg/L  | $10.72 \pm 0.59$                  | $1.27 \pm 0.12$      |
| TA-W 1000 mg/L | $10.78 \pm 0.56$                  | $1.28 \pm 0.12$      |
| TA-W 2000 mg/L | $11.11 \pm 0.52$                  | $1.32 \pm 0.12$      |
| TA-E 500 mg/L  | $9.80 \pm 0.40$                   | $1.17 \pm 0.10$      |
| TA-E 1000 mg/L | $10.90 \pm 0.83$                  | $1.30 \pm 0.15$      |
| TA-E 2000 mg/L | $11.71 \pm 0.34$                  | $1.39 \pm 0.10$      |
| Blank 2        | $10.41 \pm 0.40$                  | $1.24 \pm 0.10$      |

TA-W, *T. angustifolia* water extract; TA-E, *T. angustifolia* ethanol extract.



**Figure 2.** Power density profile of TA pollen water and ethanol extracts.

MFCs are bio-electrochemical systems that utilize microorganisms in converting organic matter into electricity. Furthermore, the microorganism (bacteria) oxidizes the organic matter and moves into an electrode that generates an electric current. MFCs are commonly utilized in treating wastewater as they offer a sustainable and energy-efficient method to provide clean water. Recently, new applications of MFCs were observed that can detect phytochemicals with ES-like structures may possess antiviral properties. Chen et al. [54] identified that the disease-treating capabilities of herbal medicines is not only exclusively determined by their antioxidant activity; instead, it also involves their electrochemical catalytic features. ES compounds display electrochemically stable intermediates during redox reactions, ensuring their resilience over successive electrochemical activities. In contrast, non-renewable antioxidant compounds scavenge free radicals but lack the abil-

ity to form stable intermediates. As a result of the irreversible oxidation, these compounds break down over long periods of electrochemical activity, making them not appropriate as sustainable catalysts [18]. In contrast, ES compounds serve as catalysts that remain stable and intact during metabolic processes, enhancing both the effectiveness and stability of drug treatments. The distinction between antioxidant and ES compounds plays a critical role in determining the therapeutic efficacy. Given the pivotal function of ES compounds in medication, as opposed to antioxidants, they hold greater potential as promising therapeutic agents.

When a plant extract demonstrates a power density ranking with an amplification factor exceeding ca. 2.0 concerning a blank, it signifies that the extract has attained a satisfactory level of electron transfer potential [3]. This observation suggests a competitive antiviral characteristic within the plant extract. The results suggest that the power density values report the electrochemical potential of TA. It is observed that it exhibited a slightly low value. Relating the results from Section 3.3, it can be hypothesized that only few compounds exhibited electrochemical activity while others did not, indicating a low bioenergy content. This result could also have an impact on its antiviral capabilities. A previous study [55] investigated and compared the raw from being put through several processing methods (i.e., stir-frying, soaking in wine or vinegar, and high-pressure steaming) of extracting the phytochemicals in *Rheum palmatum*. It revealed that different methods lead to variations in its chemical profile, influencing its bioactivity. Furthermore, stir-frying with vinegar was found to be the most appropriate method as it yielded the highest total phenolic contents, which also exhibited an increase in the amplification factors of the power density.

The increase in the antioxidant activity suggests that herbal extracts have the potential to acquire bioenergy-generating characteristics. Such findings indicated that the extracts could also persist as electrochemical catalysts in prolonged bioactivities. However, a presence of high antioxidant levels and low electron-stimulating capabilities are observed, where biological activities (e.g., antiviral) tend to occur briefly. This occurrence results in being not highly effective in preventing viral infections [3,54]. In addition, the electrochemical characteristics to be revealed as either antioxidant or ESs were also strongly influenced by the chemical structure and environmental settings. *Ortho*- and *para*-polyhydroxy substituents attached in aromatic rings could exhibit reversible and stable ES capabilities. Through inductive and resonance effects, the position of the substituents (such as *para*, *ortho*, and *meta*) on an aromatic ring influences the reactivity and regioselectivity of electrophilic (or nucleophilic) aromatic substitutions. On the other hand, flavonoid compounds, specifically those with polyhydroxy substituents in *ortho* positions, could enhance antiviral properties through synergistic effects. Considering the pathology, viral-induced oxidative stress can disrupt the host's redox balance, leading to the weakening of immune system. Consequently, bioactive compounds that are abundant in antioxidants emerge as prospective candidates for combating viral infections. Hence, the effectiveness of flavonoids for antiviral activity are linked to their antioxidant and electron-mediating properties [18,56].

#### 4. Significance of the Study

This study highlights the significance of different prospectives in shaping the pharmacological and biological properties of TA pollen. By combining the results from extraction techniques, a connection is established for assessing both the phytochemical composition and bioenergy-producing capabilities of TA pollen. Additionally, the findings indicate its potential as a promising candidate for treating serious viruses, such as dengue fever, hepatitis C, and Japanese encephalitis. Symptoms of these diseases could emerge from asymptomatic to fever, body aches, and joint pains. Neurological manifestations (e.g., seizures and convulsions) were also observed in affected children [57]. Diseases resulting from Flaviviridae exhibit severe effects since it primarily targets the central nervous system. This makes the treatment more challenging due to its impact on the immune system. People often resort to self-medication to address symptoms due to poverty, limited access, and a lack of awareness [58]. However, improper self-medication may exacerbate the condi-

tion which may result in fatal complications. Therefore, herbal medicines like TA pollen are valuable for their low cost and accessibility. Initial studies on this herbal medicine demonstrated a range of physiological effects, especially concerning blood circulation.

The results revealed that TA-W contains a higher phenolics and flavonoids content, making it the preferred solvent for the optimal extraction of these phytochemicals. In contrast, TA-E demonstrated promising ES capabilities, as evidenced by both its highest power density value (at 2000 mg/L) and the observed augmentation in its power density with an increasing concentration. Nevertheless, these values were comparatively less substantial when compared with those obtained from TA-W. In fact, the amplification factors did not surpass the 2-fold basis with respect to the blank sample, reducing the effectiveness compared to the previous studies with different plant samples [27,54]. This finding can be caused by compounds detected from the HPLC analysis. It was mentioned earlier that naturally obtained polyphenols are manifested to possess either or both antioxidant and bioenergy-generating characteristics. The electrochemical activities are likely dominated by antioxidant effects due to the selection of conditions that may be slightly less than ideal, resulting in a relatively lower bioelectricity-stimulating performance. Here, quercetin and its derivatives (quercetin-3-O-(2G- $\alpha$ -L-rhamnosyl)-rutinoside and quercetin-3-O-neohesperidoside) were the only ES compounds found in the sample. The primary aim of our research is to evaluate the antiviral activity of *Typha angustifolia* pollen (TA) during the initial screening follow-up studies to explore the interaction mechanisms between TA compounds and viral target proteins, with the goal of developing new antiviral drugs being inevitably required.

## 5. Conclusions

The research highlights the potential of TA pollen as a promising candidate in the field of drug discovery and development for illnesses induced by viral pathogens. It was found that TA-E at 2000 mg/L showed the highest power density via MFC, indicating potential for bioenergy stimulation and electron transport. This innovative electrochemical method is employed to screen potential herbal remedies with an antiviral property. The findings potentially lay the groundwork for the development of antiviral medications tailored to target diseases associated with viruses, while reducing the risk of drug toxicity. Follow-up exploration is still recommended to assess additional crucial parameters contributing to the continued advancement of effective drug development.

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## Article

# Synergistic Phytochemical and Pharmacological Actions of Hair Rise<sup>TM</sup> Microemulsion: A Novel Herbal Formulation for Androgenetic Alopecia and Hair Growth Stimulation

Anurak Muangsanguan <sup>1</sup>, Warintorn Ruksiriwanich <sup>1,2,3,\*</sup>, Pichchapa Linsaenkart <sup>1</sup>, Pensak Jantrawut <sup>1,2,3</sup>, Pornchai Rachtanapun <sup>3,4</sup>, Kittisak Jantanasakulwong <sup>3,4</sup>, Sarana Rose Sommano <sup>2,3,5</sup>, Korawan Sringarm <sup>2,3,6</sup>, Chaiwat Arjin <sup>6</sup>, Mathukorn Sainakham <sup>1</sup> and Juan M. Castagnini <sup>7</sup>

- <sup>1</sup> Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand; anurak\_m@cmu.ac.th (A.M.); pichchapa\_li@cmu.ac.th (P.L.); pensak.j@cmu.ac.th (P.J.); mathukorn.s@cmu.ac.th (M.S.)
  - <sup>2</sup> Cluster of Valorization and Bio-Green Transformation for Translation Research Innovation of Raw Materials and Products, Chiang Mai University, Chiang Mai 50200, Thailand; sarana.s@cmu.ac.th (S.R.S.); korawan.s@cmu.ac.th (K.S.)
  - <sup>3</sup> Center of Excellence in Agro Bio-Circular-Green Industry (Agro BCG), Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand; pornchai.r@cmu.ac.th (P.R.); kittisak.jan@cmu.ac.th (K.J.)
  - <sup>4</sup> School of Agro-Industry, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand
  - <sup>5</sup> Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand
  - <sup>6</sup> Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand; chaiwat.arjin@cmu.ac.th
  - <sup>7</sup> Research Group in Innovative Technologies for Sustainable Food (ALISOST), Department of Preventive Medicine and Public Health, Food Science, Toxicology and Forensic Medicine, Faculty of Pharmacy, Universitat de València, Avenida Vicent Andrés Estellés s/n, 46100 Burjassot, Spain; juan.castagnini@uv.es
- \* Correspondence: warintorn.ruksiri@cmu.ac.th

**Abstract:** Androgenetic alopecia (AGA) is a genetic condition characterized by an excessive response to androgens, leading to hairline regression in men and hair thinning at the vertex in women, which can negatively impact self-esteem. Conventional synthetic treatments for AGA are often limited by their side effects. In contrast, Thai medicinal plants offer a promising alternative with fewer adverse effects. This study investigates the synergistic phytochemical and pharmacological effects of a novel Hair Rise<sup>TM</sup> microemulsion, formulated with bioactive extracts from rice bran (*Oryza sativa*), shallot bulb (*Allium ascalonicum*), licorice root (*Glycyrrhiza glabra*), and corn kernels (*Zea mays*), for the treatment of hair loss. The microemulsion, in concentrations of 50%, 75%, and 100% (v/v), significantly enhanced the proliferation of human hair follicle dermal papilla cells (HFDPCs) compared to minoxidil. Additionally, it upregulated critical hair growth signaling pathways, including Wnt/ $\beta$ -catenin (*CTNNB1*), Sonic Hedgehog (*SHH*, *SMO*, *GLI1*), and vascular endothelial growth factor (*VEGF*), surpassing standard controls such as minoxidil and purmorphamine. The microemulsion also demonstrated potent anti-inflammatory and antioxidant properties by reducing nitric oxide production and oxidative stress, factors that contribute to inflammation and follicular damage in AGA. Furthermore, Hair Rise<sup>TM</sup> inhibited 5 $\alpha$ -reductase (types 1–3), a key enzyme involved in androgen metabolism, in both human prostate cancer cells (DU-145) and HFDPCs. These findings suggest that Hair Rise<sup>TM</sup> microemulsion presents a promising natural therapy for promoting hair growth and reducing hair loss via multiple synergistic mechanisms, offering a potent, plant-based alternative to synthetic treatments.

**Keywords:** rice bran (*Oryza sativa* L.); shallot bulb (*Allium ascalonicum* L.); licorice root (*Glycyrrhiza glabra* L.); corn kernels (*Zea mays* L.); androgenetic alopecia; Sonic Hedgehog; Wnt/ $\beta$ -catenin; angiogenesis; 5 $\alpha$ -reductase inhibition

## 1. Introduction

Androgenetic alopecia (AGA), also known as male and female pattern hair loss, is the most common type, characterized by progressive terminal hair loss after middle age [1]. In general, the major phase of hair follicle formation is composed of the stages of growth (anagen), regression (catagen), resting (telogen), and shedding (exogen), influenced by numerous factors [2]. Indeed, the major common risk in AGA is an androgen hormone. Dihydrotestosterone (DHT) is an androgenic steroid hormone induced by the action of 5 $\alpha$ -reductase (SRD5A) enzymes, which convert testosterone (TT) to DHT. The overproduction of DHT in androgen-dependent areas may reduce the growth stage in the hair cycle, leading to the shrinkage of the hair follicle and finally hair loss [3]. In addition, inflammation in hair follicles is recognized as a contributing factor in the development of AGA [4]. Oxidative stress and DHT have been shown to elevate nitric oxide (NO) levels in hair follicles [5].

In contrast, the formation of hair follicles requires interaction between the mesenchymal cells and epithelial cells, which receive signaling from human hair follicle dermal papilla cells (HFDPCs), including Wnt/ $\beta$ -catenin (CTNNB1), Sonic Hedgehog (SHH, SMO, and GLI1), and vascular endothelial growth factor (VEGF) signaling pathways [6]. The CTNNB1 signaling pathway is a key pathway for hair growth formation. The stimulation of  $\beta$ -catenin in HFDPCs at the hair germ and hair bulge initiates the lymphoid enhancer factor (LEF) or T-cell factor (TCF) complex and initiates the transcription of downstream target genes such as c-Myc and cyclin D1. This process stimulates the proliferation, migration, and differentiation of HFDPCs in the early stages of the anagen phase [7]. Moreover, CTNNB1 acts as an upstream regulator of the Sonic Hedgehog signaling pathway, which stimulates the development of hair follicles in the middle stage of the anagen stage by promoting the proliferation and migration of epithelial and mesenchymal cells [7,8]. In addition, VEGF supports angiogenesis in the growth stage, enhancing the delivery of nutrient- and oxygen-rich blood to the hair follicles [9].

The FDA-approved treatments for hair loss include topical minoxidil, oral finasteride, and low-level laser therapy [10]. Minoxidil is converted into its active form (minoxidil sulfate), which activates adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channels, leading  $K_{ATP}$  channels to open. This mechanism makes minoxidil a  $K_{ATP}$  channel opener, leading to hyperpolarization of cell membranes, dilation of vascular smooth muscles, and increased blood circulation. The opening of  $K_{ATP}$  channels stimulates the growth of HFDPCs, contributing to hair follicle development and stimulating new hair [11]. Finasteride is an inhibitor of the SRD5A type 2 isoenzyme, which was developed to treat benign prostatic hyperplasia and is also used to treat AGA [12]. Furthermore, several treatments, including cell-based treatments, laser therapy, and natural cosmetic product treatments, are being used to treat AGA. The development of novel green cosmetic products containing phytochemicals from natural sources has significantly increased because they are less toxic and more environmentally friendly than conventional cosmetics.

Plants generate constitutive metabolites known as phytochemicals, which are essential to their survival and functionality. Phytochemicals are classified into various categories, such as polyphenols (including phenolic and flavonoid compounds), alkaloids, terpenoids, oryzanols, and tocopherols, each exhibiting diverse biochemical and biological activities based on their chemical structures. [13]. Furthermore, various studies have highlighted the potential of phytochemicals as agents against hair loss by activating hair growth factors such as CTNNB1, Hedgehog, and VEGF, and inhibiting hair loss stimulators like SRD5A, transforming growth factor (TGF- $\beta$ 1), and Dickkopf-related protein 1 (DKK-1) [14]. Rice, also known as *Oryza sativa* L., is extensively consumed on a global scale. The composition of rice grains consists of rice husk and rice bran. Rice bran is the most common by-product of rice milling, accounting for approximately 20% of the total weight of whole rice [15]. Previous studies have indicated that BB3-CMU and BB4-CMU rice bran, which is cultivated in northern Thailand, contains a variety of minerals and bioactive compounds, such as polyphenols (phenolic and flavonoid compounds), oryzanols, and tocopherols [15]. Additionally, shallot bulbs (*Allium ascalonicum* L.) are an economically essential herb in

Thailand and are used as a diet and traditional medicine in Asia. Shallot was used for its various pharmacological properties, such as antimicrobial, anticancer, anti-inflammatory, and antioxidant effects [16]. Our previous study showed that shallot extracts contained a high level of polyphenols, including *p*-coumaric acid, quercetin, and rosmarinic acid. Furthermore, our study confirmed the potential of rice bran and shallot extract in terms of their anti-inflammatory activities and their modulation of *SRD5A* gene expression [16–18]. Licorice root (*Glycyrrhiza glabra* L.) has been widely used in traditional medicine for centuries, particularly in Asia, including India and China [19]. It contains bioactive compounds such as polyphenols, chromenes, coumarins, and glycyrrhizic acid, with polyphenols and glycyrrhizic acid being the major constituents [20]. Glycyrrhizic acid promotes hair growth by enhancing scalp conditions through its anti-inflammatory properties, which indirectly support hair growth [21]. Zhang et al. reported that glycyrrhizic acid in licorice root could induce the stimulation of hair growth factors and angiogenesis activities in HFDPCs, resulting in hair follicle development and finally hair growth [22]. Additionally, glycyrrhizic acid has been shown to inhibit dihydrotestosterone (DHT) production by suppressing the expression of *SRD5A* genes [21]. Guava (*Psidium guajava* L.) is used as food and traditional medicine due to its pharmacological properties [23]. It is rich in bioactive compounds, particularly polyphenols, which contribute to its antioxidant and anti-inflammatory activities. In Southwest Asia, guava has traditionally been used to stimulate hair growth [23]. Additionally, our previous study demonstrated that guava leaf extract can inhibit the expression of the *SRD5A* gene, which is associated with hair loss [24]. Lastly, corn kernels (*Zea mays* L.), are an essential source of nutrition for humans and animals worldwide. It has been widely used in traditional herbal medicine to treat various diseases, including obesity, edema, and gout [25]. Corn kernels are rich in a variety of phytochemicals, including polyphenols, polysaccharides, proteins, vitamins, and alkaloids, and particularly ferulic acid, which has beneficial antioxidant and anti-inflammatory effects in hair development [26]. All extracts were prepared in the form of microemulsions to increase the stability and skin penetration efficiency of active ingredients. Microemulsion is a drug delivery system composed of water and oil phases stabilized by a combination of surfactant and cosurfactant. The particle size of the microemulsion is less than 100 nm, which achieves optimum effects and offers advantages such as temperature stability, translucency, and long-term settling [27].

As previously mentioned, the bioactive compounds in selected medicinal plant extracts with high potential biological activities were formulated as a multipurpose hair tonic microemulsion, commercially named 'Hair Rise<sup>TM</sup>'. Therefore, this study aims to estimate the potential of a Hair Rise<sup>TM</sup> microemulsion containing medicinal plant extracts for treating hair loss and promoting hair growth. The antioxidant, anti-inflammatory, and gene expression regulation involving androgen, Wnt/ $\beta$ -catenin, Sonic Hedgehog, and angiogenesis signaling pathways for AGA treatment of Hair Rise<sup>TM</sup> microemulsion were determined.

## 2. Results and Discussion

### 2.1. Extraction Yield and Bioactive Compound Estimation

For the preparation of the BB3-CMU rice bran and shallot extracts by ScCO<sub>2</sub>, the extraction conditions were set at a fixed co-solvent, pressure, temperature, and time: 95% (*v/v*) ethanol, 400 bar, 50 °C, and 0.5 h, respectively. These conditions were established based on findings from our previous research [28]. Our previous study revealed that high pressure (400 bar) significantly enhances extraction yield compared to lower pressures because the pressure influences the density of carbon dioxide. Increased pressure raises the carbon dioxide density within the extraction column, enhancing the penetration of carbon dioxide and any co-solvents through the material. As a result, this increased density leads to better extraction performance and higher extraction yields [28]. Furthermore, the addition of polar modifiers, such as 95% (*v/v*) ethanol as co-solvents in the ScCO<sub>2</sub> process, could increase the solubility of target compounds in the mixture, thereby improving extraction yields simultaneously [28]. The extraction temperature was fixed at 50 °C because



of concerns about the stability of bioactive constituents in the extract. As the temperature increased between 40 and 60 °C, the temperature at 50 °C showed the highest bioactive constituent content [29]. In this study, the BB3-CMU rice bran extracts have a dark green-brown semisolid and greasy texture, with an extraction yield of  $14.80 \pm 0.04\%$  (*w/w*) based on the dry materials (Table 1). The BB3-CMU rice bran extract showed the highest content of polyphenols ( $32.42 \pm 1.42$  mg GAE/g extract for total phenolic content and  $56.03 \pm 1.14$  mg EGCG/g extract for total flavonoid content). These results align with previous studies indicating that BB3-CMU rice bran showed high contents of polyphenols [15]. The successful extraction of these compounds from BB3-CMU rice bran is influenced by various factors, such as extraction temperature, co-solvent type, pressure, and degree of mechanical agitation [30]. These factors contribute to achieving a greater concentration of bioactive compounds in the extracts. The three main bioactive compounds found in BB3-CMU rice bran were  $\alpha$ -tocopherol ( $8.54 \pm 0.02$  mg/g extract),  $\gamma$ -tocopherol ( $3.65 \pm 0.01$  mg/g extract), and chlorogenic acid ( $0.11 \pm 0.01$  mg/g extract). Our results were consistent with previously established findings that tocopherol was primarily evident in rice bran [18]. A recent study reported that tocopherol could induce the expression of genes related to hair growth, especially Wnt/ $\beta$ -catenin activity in HFDPCs. This activation contributes to an extended growth phase in the hair cycle and encourages new hair development [17]. Chlorogenic acid has been reported to be the main phenolic compound in rice bran, rice husk, and grain [15]. Furthermore, chlorogenic acid has been reported to have anti-hair loss activities via the anti-androgen pathway by inhibiting the expression of the *SRD5A* gene and stimulating new hair via the inducer growth factors' gene expression in the hair follicle formation process [28]. In addition, a previous study indicated that unsaturated fatty acids (oleic acid and  $\gamma$ -linolenic acid) found in rice bran extract significantly suppressed *SRD5A* expression in HFDPCs [2].

**Table 1.** Bioactive compounds and antioxidant activities of plant extracts.

| Results                                     | Plant Extract     |                  |                  |                  |
|---------------------------------------------|-------------------|------------------|------------------|------------------|
|                                             | BB3-CMU Rice Bran | Shallot Bulb     | Licorice Root    | Corn Kernels     |
| Extraction yield (%)                        | $14.80 \pm 0.04$  | $5.85 \pm 0.09$  | $11.21 \pm 0.24$ | $22.41 \pm 0.86$ |
| Total phenolic content (mg GAE/g extract)   | $32.42 \pm 1.42$  | $4.18 \pm 0.03$  | $3.21 \pm 0.12$  | $14.42 \pm 0.56$ |
| Total flavonoid content (mg EGCG/g extract) | $56.03 \pm 1.14$  | $2.54 \pm 0.01$  | $1.96 \pm 0.08$  | $4.33 \pm 0.12$  |
| Chlorogenic acid (mg/g extract)             | $0.11 \pm 0.01$   | -                | $0.12 \pm 0.01$  | $4.22 \pm 0.51$  |
| <i>p</i> -Coumaric acid (mg/g extract)      | $0.07 \pm 0.01$   | $1.02 \pm 0.01$  | -                | $0.33 \pm 0.01$  |
| Rosmarinic acid (mg/g extract)              | $0.05 \pm 0.01$   | $0.21 \pm 0.01$  | -                | -                |
| Ferulic acid (mg/g extract)                 | $0.02 \pm 0.01$   | -                | -                | $5.14 \pm 0.01$  |
| Epigallocatechin gallate (mg/g extract)     | $0.03 \pm 0.03$   | -                | $0.09 \pm 0.02$  | -                |
| Quercetin (mg/g extract)                    | $0.03 \pm 0.01$   | $0.03 \pm 0.01$  | $0.07 \pm 0.01$  | -                |
| $\alpha$ -Tocopherol (mg/g extract)         | $8.54 \pm 0.02$   | -                | -                | -                |
| $\gamma$ -Tocopherol (mg/g extract)         | $3.65 \pm 0.01$   | -                | -                | -                |
| Glycyrrhizic acid (mg/g extract)            | -                 | -                | $0.62 \pm 0.02$  | -                |
| Antioxidant activities (%)                  | ABTS              | $32.14 \pm 2.03$ | $21.16 \pm 1.33$ | $16.15 \pm 0.21$ |
|                                             | DPPH              | $28.89 \pm 1.08$ | $19.15 \pm 0.96$ | $14.16 \pm 0.24$ |

Note: Values are presented as the mean  $\pm$  standard deviation (SD). Milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract); milligrams of epigallocatechin gallate equivalents per gram of extract (mg EGCG/g extract); milligrams per gram of extract (mg/g extract).

The shallot extract appeared greasy, dark gray, and semisolid. The extraction yield was  $5.85 \pm 0.09\%$  (*w/w*) based on dry material. The total phenolic and flavonoid content of shallot was  $4.18 \pm 0.03$  mg GAE/g extract and  $2.54 \pm 0.01$  mg EGCG/g extract, respectively (Table 1). The phenolic compounds found in shallot extract were *p*-coumaric acid ( $1.02 \pm 0.01$  mg/g extract), followed by rosmarinic acid ( $0.21 \pm 0.01$  mg/g extract), and quercetin ( $0.03 \pm 0.01$  mg/g extract). Our results were consistent with previous findings that shallot extract contains *p*-coumaric acid [31]. Moreover, Khantham et al. reported the



potential of shallot extracts for their beneficial effects on the stimulation of hair growth, including anti-androgenic and anti-inflammation pathways [16].

The corn kernels and licorice root were extracted by conventional maceration (CE). CE is a widely used method for extracting bioactive compounds with recognized biological activities from medicinal plants [32]. In this study, the extraction yield of corn kernels ( $22.41 \pm 0.86\%$  (*w/w*) based on dry material) was higher than that of licorice root ( $11.21 \pm 0.24\%$  (*w/w*) based on dry material) (Table 1). The physical appearance of corn kernel extract was dark yellow, semisolid, and greasy. Licorice root is dark brown, semisolid, and greasy. The phenolic and flavonoid compounds of licorice root extract were  $3.21 \pm 0.12$  mg GAE/g extract and  $1.96 \pm 0.08$  mg EGCG/g extract, respectively. The primary bioactive compound in licorice root was glycyrrhizic acid ( $0.62 \pm 0.02$  mg/g extract). Glycyrrhizic acid is recognized for its steroid-like properties and strong pharmacological actions, such as anti-inflammatory, anti-gastric ulcer, anti-hepatotoxic, and antiviral activities [33]. In addition, Zhang et al. reported the potential of glycyrrhizic acid at different concentrations (1%, 10%, and 20% (*v/v*) in water) on hair growth in mice compared to minoxidil, a standard treatment for anti-hair loss. The results showed that glycyrrhizic acid at 10% and 20% (*v/v*) in water stimulated hair density in mice higher than minoxidil [22]. Moreover, glycyrrhizic acid enhanced the expression of Wnt/ $\beta$ -catenin and angiogenesis activities in HFDPCs, higher than minoxidil [22]. Lastly, the phenolic and flavonoid contents of the corn kernel extract were  $14.42 \pm 0.56$  mg GAE/g extract and  $4.33 \pm 0.12$  mg EGCG/g extract, respectively. Ferulic acid ( $5.14 \pm 0.01$  mg/g extract), chlorogenic acid ( $4.22 \pm 0.51$  mg/g extract), and *p*-coumaric acid ( $0.33 \pm 0.01$ ) were detected. Ferulic acid has been reported to exhibit various pharmacological activities, particularly antioxidant and anti-inflammatory effects in the hair cycle [26]. Furthermore, Xu et al. reported the potential of ferulic acid to enhance mRNA expression in DHT-irritated HFDPCs and stimulate hair growth in mice with androgenetic alopecia [34].

## 2.2. Antioxidant Activities of Plant Extracts

The antioxidant properties of all plant extracts were evaluated using the ABTS and DPPH scavenging assays (Table 1). Among the tested extracts, the BB3-CMU rice bran extract exhibited the strongest antioxidant activity, with values of  $32.14 \pm 2.03\%$  and  $28.89 \pm 1.08\%$  for ABTS and DPPH radicals, respectively. The high level of polyphenol compounds significantly contributes to their strong antioxidant activities [35]. Polyphenols are known for their antioxidant properties due to a high number of hydroxyl groups in their structures [36]. Therefore, BB3-CMU rice bran not only contained the highest polyphenol content, but also demonstrated superior efficacy in neutralizing ABTS and DPPH radicals.

As previously described, all plant extracts demonstrated high levels of bioactive compounds. These compounds can stimulate hair growth through various pathways, including antioxidant, anti-inflammatory, and anti-androgenetic pathways, which prolong the duration of the anagen stage of the hair cycle. Additionally, these compounds can activate hair growth pathways such as Wnt/ $\beta$ -catenin, Sonic Hedgehog, and angiogenesis pathways, which support hair growth through prolonging the anagen stage and stimulating blood flow to the hair follicles. Based on our data, all plant extracts were selected for the development of a Hair Rise<sup>TM</sup> microemulsion formulation.

## 2.3. Characterization of the Hair Rise<sup>TM</sup> Microemulsion

The physical stability of the Hair Rise<sup>TM</sup> microemulsion was determined by observing changes in particle size, or Z-average (Z-Ave), polydispersity index (pdI), and zeta potential (ZP) values after 3 months of storage at room temperature. After 3 months of storage, the Z-Ave of Hair Rise<sup>TM</sup> microemulsion at initial, 1, 2, and 3 months demonstrated values of  $30.57 \pm 0.17$ ,  $32.43 \pm 0.06$ ,  $38.96 \pm 1.32$ , and  $48.38 \pm 0.88$  nm, respectively. Moreover, after six cycles of heating-cooling acceleration, the Z-Ave showed a value of  $42.43 \pm 0.91$  nm. Microemulsions are small colloidal dispersion systems with particle sizes ranging from 10 to 100 nm. The small particle size could provide advantages in the

Hair Rise™ formulation, such as thermal stability, translucency, and long-term stability. Furthermore, pdI, defined as the ratio of the standard deviation to the average particle size, indicates the uniformity of the particle sizes. The low pdI value (<0.4 nm) demonstrates the high uniformity of the particle sizes in the Hair Rise™ formulation [37].

The ZP is a crucial characteristic of particles that influences both their stability and ability to adhere to cells. The ZP, whether positive or negative, plays a vital role in maintaining the stability of particle suspensions. This stability is primarily due to the electrostatic repulsion observed among particles with similar charges, which results in their separation. Our results showed that the Hair Rise™ microemulsion was highly stable when stored for 3 months (Table 2). The high stability of the Hair Rise™ microemulsion indicates that it functions as a thermodynamically stable system [38].

**Table 2.** Physical stability of Hair Rise™ microemulsion after storage for 3 months and six cycles of heating–cooling acceleration.

| Condition                                              | Size (nm)                 | Zeta Potential (mV)       | pdI                      |
|--------------------------------------------------------|---------------------------|---------------------------|--------------------------|
| At initial                                             | 30.57 ± 0.17 <sup>a</sup> | −3.36 ± 0.05 <sup>a</sup> | 0.23 ± 0.02 <sup>b</sup> |
| After 1 month                                          | 32.43 ± 0.06 <sup>a</sup> | −1.87 ± 0.03 <sup>b</sup> | 0.11 ± 0.01 <sup>a</sup> |
| After 2 months                                         | 38.96 ± 1.32 <sup>b</sup> | −0.27 ± 0.01 <sup>e</sup> | 0.26 ± 0.02 <sup>c</sup> |
| After 3 months                                         | 48.38 ± 0.88 <sup>d</sup> | −0.34 ± 0.02 <sup>d</sup> | 0.21 ± 0.03 <sup>b</sup> |
| After six cycles of heating–cooling accelerate process | 42.43 ± 0.91 <sup>c</sup> | −0.66 ± 0.02 <sup>c</sup> | 0.23 ± 0.03 <sup>b</sup> |

Note: Values are presented as the mean ± SD for triplicates in each sample. Statistical analyses were performed using one-way ANOVA, followed by Tukey's HSD test, with significance defined at  $p < 0.05$  in each sample. Statistical differences are represented by different letters (a–e).

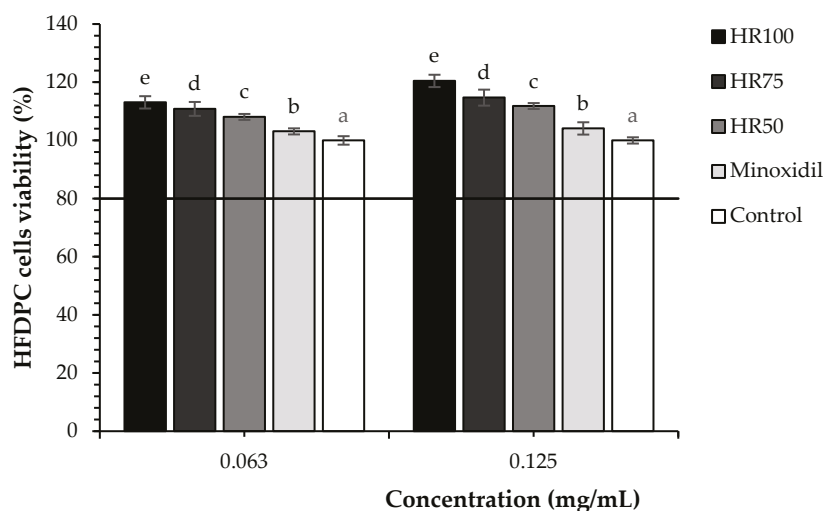
#### 2.4. Effect of Hair Complex Microemulsion on Cell Viability and Proliferation

The effects of plant extracts and Hair Rise™ microemulsions on cell viability and proliferation were assessed across concentrations from 0.0625 to 2 mg/mL in RAW 264.7, DU-145, and HFDPCs. According to ISO 10993-5 guidelines, a cell survival rate exceeding 80% is regarded as non-toxic [39]. After 24 h of treatments, plant extracts (BB3-CMU rice bran, shallot bulb, licorice root, and corn kernels) at a concentration  $\geq 0.5$  mg/mL demonstrated cytotoxicity and significantly decreased the viability of all types of cells compared to untreated cells. The maximum concentration of plant extract (0.25 mg/mL) that maintained cell viability above 80% was classified as non-toxic.

In the formulation part, all concentration (50, 75, and 100% (v/v) in water) of Hair Rise™ microemulsion at a concentrations  $\geq 0.5$  mg/mL in all cells showed no cytotoxicity at the comparable level of untreated cells ( $p > 0.01$ ). So, the highest concentration of 0.5 mg/mL, which gave viability above 80%, was selected for further experiments. Furthermore, all of the different concentrations of Hair Rise™ microemulsion significantly increased the proliferation of HFDPCs at concentrations of 0.063 and 0.125 mg/mL compared with the untreated cells ( $p > 0.01$ ) (Figure 1). Moreover, the proliferation of HFDPCs at all concentrations (50, 75, and 100% (v/v) in water) of Hair Rise™ microemulsion was higher than that of minoxidil. As is known, minoxidil could promote the cell viability of HFDPCs by activating the ERK and AKT signaling pathways, preventing cell death, and increasing the cell viability of HFDPCs [40,41].

HFDPCs play a vital role in hair follicle formation and the stimulation of hair growth [42]. The process of hair follicle formation consists of three primary stages: induction, organogenesis, and cytodifferentiation, and these three stages include eight steps [43]. Firstly, HFDPCs generate signaling for hair follicle cell regeneration, which induces the proliferation, migration, and differentiation of epithelial cells, finally forming the hair follicle basal plate. After that, the hair follicle basal plate sends the signal back to the HFDPCs to stimulate the aggregate of HFDPCs and lead to the formation of the new hair follicle. In the second step, aggregation of the HFDPCs generates the signaling to induce the expansion of the hair follicle basal plate downward to enter the dermis and form the hair buds. Then, the hair buds expand and elongate in the dermis, and the fibroblast cells in the dermis

translocate to the hair buds. Afterward (third step), HFDPCs induce the proliferation of keratinocyte cells. These keratinocyte cells overlap and form a column around the hair buds. In the fourth step, HFDPCs under the hair follicle basal plate translocate to the hair follicle basal plate and form dermal papillae. The fourth and the fifth steps occur at the same time; HFDPCs induce the proliferation and migration of the hair matrix, leading to the differentiation of hair matrix cells to the transit-amplifying progenitor cells and the formation of the hair shaft and inner root sheath, respectively. In the sixth step, the accessory organ of the hair follicle structure is completely developed by the formation of epithelial cells. In the seventh step, the hair follicle elongates and moves through the hair canal. Finally (eighth step), the hair follicle is completely formed, and the hair shaft enters through the epidermis [43–45].



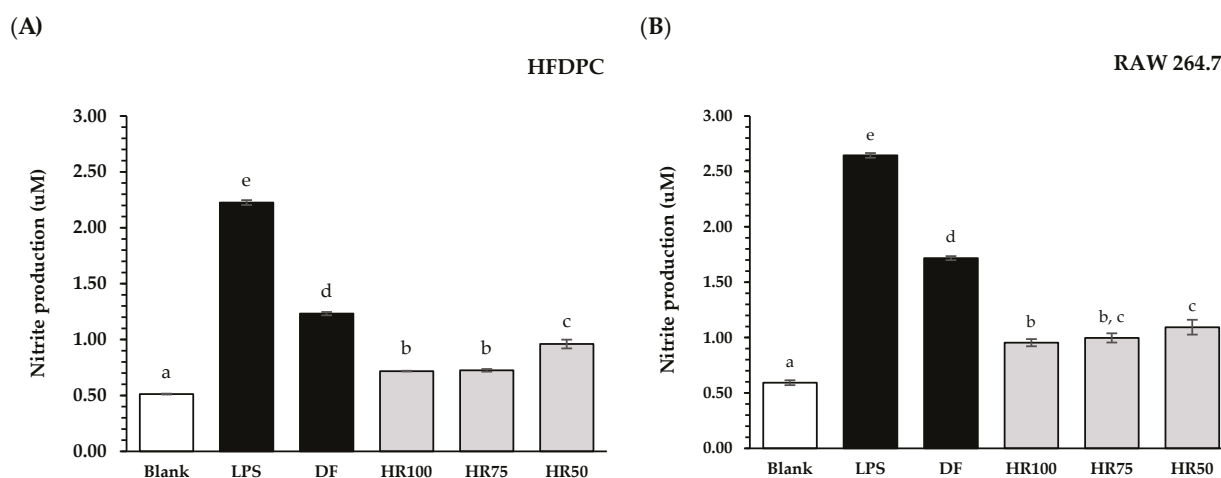
**Figure 1.** The effect of Hair Rise<sup>TM</sup> microemulsions and standard controls (minoxidil) at concentrations of 0.063 and 0.125 mg/mL on the proliferation of HFDPCs was compared to untreated cells. Values were presented as the mean  $\pm$  SD for measurements for each group. A one-way ANOVA was used for statistical analysis, followed by Tukey's HSD test. Statistical differences ( $p < 0.05$ ) in cell proliferation among samples at each treatment concentration (0.063 and 0.125 mg/mL) are indicated by different letters (a–e). HR100: 100% of Hair Rise<sup>TM</sup> microemulsion; HR75: Hair Rise<sup>TM</sup> microemulsion at a concentration of 75% (v/v) in water; HR50: Hair Rise<sup>TM</sup> microemulsion at a concentration of 50% (v/v) in water.

As previously described, HFDPCs play an essential role in all steps of hair follicle development. This study indicated that all Hair Rise<sup>TM</sup> microemulsion samples at concentrations of 0.063 and 0.125 mg/mL promoted HFDPC proliferation, which may promote the growth of hair follicles, leading to hair growth stimulation [42]. Moreover, high concentrations of Hair Rise<sup>TM</sup> microemulsion showed greater HFDPC proliferation compared to lower concentrations in the cell proliferation assay (0.063 and 0.125 mg/mL). These results demonstrate that a higher concentration of Hair Rise<sup>TM</sup> microemulsion enhances HFDPC proliferation more effectively than a lower concentration [46]. This result may be attributed to the synergistic effects of compounds present in the Hair Rise<sup>TM</sup> microemulsion, such as vitamin B5, and the bioactive compounds in plant extracts, including ferulic acid,  $\alpha$ -tocopherol, and other components. These compounds align with previous studies showing that ferulic acid,  $\alpha$ -tocopherol, and vitamin B5 can stimulate the proliferation of HFDPCs and promote hair growth. [17,47,48].

## 2.5. Effect of Hair Complex Serum on Anti-Inflammatory Activities

Increased NO levels have been reported to impact hair regeneration [4]. The inflammation in hair follicles is induced by oxidative stress and DHT [5]. Previous studies reported that the NO level in HFDPCs of patients with androgenetic alopecia was higher

than that of healthy volunteers [49]. In this study, all concentrations (50%, 75%, and 100% (v/v) in water) of Hair Rise<sup>TM</sup> microemulsion and the standard drugs (diclofenac sodium) at a concentration of 0.5 mg/mL were evaluated on HFDPCs and RAW 264.7 cells to compare their inhibitory effects on NO production. NO production was induced by lipopolysaccharide (LPS), an inflammatory stimulator derived from *Escherichia coli* [50]. As demonstrated in Figure 2, both in HFDPCs and RAW 264.7 cells, the level of nitrite produced by the LPS-stimulated group without any prior treatment was higher than that of the solvent-treated control group (blank). Moreover, all tested samples significantly suppressed nitrite production compared to the nitrite level of the LPS-stimulated group ( $p < 0.05$ ). As a result, all concentrations (50%, 75%, and 100% (v/v) in water) of Hair Rise<sup>TM</sup> microemulsion showed significantly higher nitrite suppression than diclofenac sodium (a standard inflammatory drug) in both HFDPCs and RAW 264.7 cells ( $p < 0.05$ ). In comparison to Hair Rise<sup>TM</sup> microemulsion at different concentrations (50%, 75%, and 100% (v/v) in water), there was no significant difference between the nitrite level of 75% (v/v) in water and 100% of Hair Rise<sup>TM</sup> microemulsion. Interestingly, the lowest concentration of Hair Rise<sup>TM</sup> microemulsion (50% (v/v) in water) showed higher suppression than the diclofenac sodium in both HFDPCs and Raw 264.7 cells. Hair Rise<sup>TM</sup> microemulsion contains several compounds, including vitamin B5, which comply with previous research indicating that these substances can promote hair growth by suppressing nitrite production in hair follicle cells [51,52]. Furthermore, the bioactive compounds in rice bran, shallot bulb, licorice root, and corn kernels have been reported, such as  $\alpha$ -tocopherol,  $\gamma$ -oryzanol, glycyrrhizic acid, and polyphenols, which could suppress LPS-induced nitrite production [16,17,22,53]. Plant extracts in the formulation of Hair Rise<sup>TM</sup> microemulsion contain many anti-inflammation compounds that could suppress nitrite production and decrease inflammatory-induced perifollicular damage in hair loss.

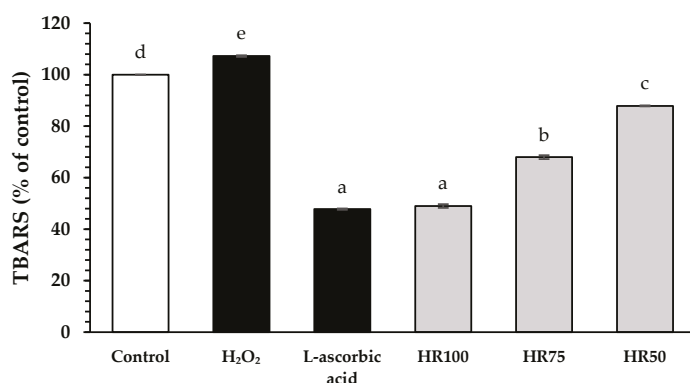


**Figure 2.** Effects of Hair Rise<sup>TM</sup> microemulsions and standard controls (diclofenac sodium) at 0.5 mg/mL on nitrite level in the lipopolysaccharide (LPS)-induced HFDPCs (A) and RAW 264.7 (B) cells for 24 h compared to untreated cells (blank) and LPS-induced control (+LPS). DF: diclofenac sodium; HR100: 100% Hair Rise<sup>TM</sup> microemulsion; HR75: Hair Rise<sup>TM</sup> microemulsion at a concentration of 75% (v/v) in water; HR50: Hair Rise<sup>TM</sup> microemulsion at a concentration of 50% (v/v) in water. Values are presented as the mean  $\pm$  SD for triplicates in each sample. A one-way ANOVA was used for statistical analysis, followed by Tukey's HSD test. Statistical differences ( $p < 0.05$ ) are represented by different letters (a–e).

## 2.6. Effect of Hair Rise<sup>TM</sup> Microemulsion on Antioxidant Activities in HFDPCs

Malondialdehyde is a by-product generated by the lipid peroxidation process. The reaction between lipid peroxidation and thiobarbituric acid produces a dark pink compound that can be detected by a UV–Vis spectrophotometer [54]. The thiobarbituric acid reactive substances (TBARS) method was used to determine the malondialdehyde production in

hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-stimulated HFDPCs, as demonstrated in Figure 3. The antioxidant activities of all concentrations (50%, 75%, and 100% (v/v) in water) of Hair Rise<sup>TM</sup> microemulsion were compared to the standard control L-ascorbic acid ( $47.81 \pm 0.24\%$  of control) as a positive control. The TBARS level of hydrogen peroxide-induced HFDPCs ( $107.26 \pm 0.32\%$  of control) was significantly different from the untreated cells (control group) ( $p < 0.05$ ). All concentrations (50%, 75%, and 100% (v/v) in water) of Hair Rise<sup>TM</sup> microemulsion showed antioxidation activities with decreased TBARS levels at  $87.88 \pm 0.19\%$ ,  $68.01 \pm 0.74\%$ , and  $49.03 \pm 0.73\%$  of control, respectively. Interestingly, 100% Hair Rise<sup>TM</sup> microemulsion showed a comparable TBARS level to L-ascorbic acid (positive control) at  $p < 0.05$ . A previous study reported that TBARS levels were significantly higher in AGA patients compared to that of the healthy group, whereas SOD and GSH levels were significantly lower in AGA patients than those in the healthy group [55].



**Figure 3.** Effects of Hair Rise<sup>TM</sup> microemulsions and standard controls (L-ascorbic acid) at the concentration of 0.5 mg/mL on the malondialdehyde production in hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-induced HFDPCs using the thiobarbituric acid reactive substances (TBARS) assay. HR100: 100% Hair Rise<sup>TM</sup> microemulsion; HR75: Hair Rise<sup>TM</sup> microemulsion at a concentration of 75% (v/v) in water; HR50: Hair Rise<sup>TM</sup> microemulsion at a concentration of 50% (v/v) in water. Values are presented as the mean  $\pm$  SD for triplicates in each sample. A one-way ANOVA was used for statistical analysis, followed by Tukey's HSD test. Statistical differences ( $p < 0.05$ ) are represented by different letters (a–e).

Hair Rise<sup>TM</sup> microemulsion contains several antioxidant substances, such as vitamin B5. Furthermore, vitamin B5 plays an important role in protecting cells from ROS by regulating the synthesis of acetyl coenzyme A, which is important for several cellular processes. Specifically, vitamin B5 can remove lipid peroxidation products, enhance cell membrane repair mechanisms, and synthesize cholesterol [56]. In addition, selected medicinal plant extracts and specific compounds with antioxidant and anti-inflammatory effects play a crucial role in the hair follicle. The bioactive compounds in Hair Rise<sup>TM</sup> microemulsion play an important role as free radical scavengers, suppressors of radical chain reactions and oxidative enzymes, metal chelators, and stimulators of antioxidant enzymes, including CAT, SOD, and GSH, that could protect the hair follicle against ROS under oxidative stress [57].

### 2.7. Effects of Hair Rise<sup>TM</sup> Microemulsion on Gene Expression

Hair regeneration is a complex process. Generally, the follicle's major stages of the hair cycle include growth, regression, resting, and shedding, all influenced by various factors [2]. The main regulatory pathways of hair regeneration, including the androgen, Wnt/ $\beta$ -catenin, Sonic Hedgehog, and angiogenesis signaling pathways, were evaluated in this study [42]. DHT is an androgenic steroid hormone induced by the action of SRD5A enzymes, which convert testosterone to DHT. The overproduction of DHT in androgen-dependent areas may shorten the growth stage in the hair cycle, resulting in the early shrinkage of the hair follicle, and finally hair loss [3]. Hair Rise<sup>TM</sup> microemulsion contains



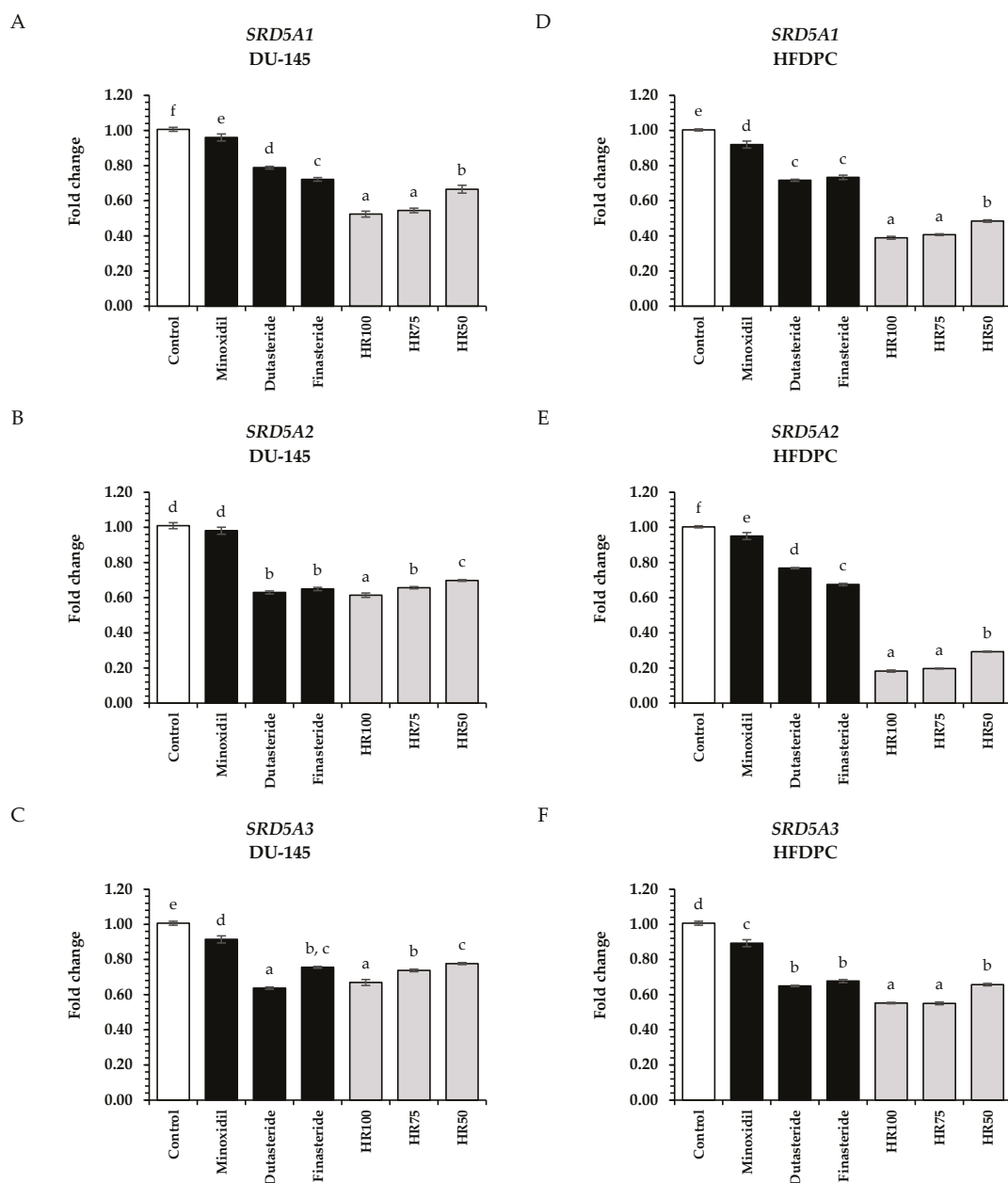
several compounds, including tocopherol, vitamin B5, ferulic acid, and various plant extracts. Previous research indicated that  $\alpha$ -tocopherol and  $\gamma$ -oryzanol in rice bran played an essential role in SRD5A enzyme inhibition (Table 3) [17,18]. Moreover, shallot extract contains a lot of *p*-coumaric acid, rosmarinic acid, and quercetin, which also suppresses SRD5A activity, similar to glycyrrhizic acid in licorice root [16,58]. Initially, we evaluated the effects of Hair Rise<sup>TM</sup> microemulsions on the expression of genes associated with the androgen pathway (*SRD5A1*, *SRD5A2*, and *SRD5A3*) in DU-145 cells and HFDPCs. The Hair Rise<sup>TM</sup> microemulsion and standard controls, including minoxidil, dutasteride, and finasteride, were determined at 0.5 mg/mL. The suppression effects of Hair Rise<sup>TM</sup> microemulsion on genes associated with the androgen pathway are shown in Figure 4. All of the Hair Rise<sup>TM</sup> microemulsions significantly inhibited the expression of *SRD5A1*, *SRD5A2*, and *SRD5A3* compared to the untreated cells (control group) and standard controls (minoxidil, dutasteride, and finasteride) in both DU-145 cells and HFDPCs ( $p < 0.05$ ). The 100% Hair Rise<sup>TM</sup> microemulsion showed the highest *SRD5A1*, *SRD5A2*, and *SRD5A3* gene suppression at  $0.52 \pm 0.02$ ,  $0.61 \pm 0.01$ , and  $0.67 \pm 0.02$  in DU-145 cells and  $0.39 \pm 0.01$ ,  $0.18 \pm 0.01$ , and  $0.55 \pm 0.01$  in HFDPCs, respectively, ( $p < 0.05$ ).

The *SRD5A* gene expression of Hair Rise<sup>TM</sup> microemulsion demonstrated in a concentration-dependent manner in both DU-145 and HFDPCs. However, the 75% (*v/v*) of Hair Rise<sup>TM</sup> microemulsion showed no significant difference in the *SRD5A* genes suppression compared to the 100% of Hair Rise<sup>TM</sup> microemulsion in both DU-145 and HFDPCs ( $p > 0.05$ ). Furthermore, the Hair Rise<sup>TM</sup> microemulsion showed the inhibition of *SRD5A* in HFDPCs, which are key to hair shaft development and play a central role in promoting hair growth, compared to DU-145 cells, a prostate cancer cell line commonly used to study 5 $\alpha$ -reductase isoenzymes.

HFDPCs are responsible for the formation of hair follicles and the production of hair growth. As previously described, the formation of hair follicles requires interaction between the mesenchymal cells and epithelial cells, which receive signaling from HFDPCs [6]. The Wnt/ $\beta$ -catenin (*CTNNB1*) signaling pathway is central to HFDPC proliferation, where Wnt proteins bind to Frizzled receptors and a low-density lipoprotein-related protein (LRP) or tyrosine kinase receptors, stabilizing  $\beta$ -catenin to prevent degradation. This activates downstream genes responsible for cell proliferation and migration, supporting hair growth [59,60].

The Wnt/ $\beta$ -catenin signaling pathway is an upstream process of the Sonic Hedgehog signaling pathway [7]. The Sonic Hedgehog signaling pathway is pivotal for signal transduction between mesenchymal and epithelial cells and plays a role in intracellular regulation. Finally, this pathway stimulates the development of the epidermis and hair follicles, repairs damage, and maintains the characteristics of hair follicle bulge stem cells. In the canonical Sonic Hedgehog signaling pathway, Sonic Hedgehog (SHH) ligands bind to the transmembrane receptor protein, or Patched (PTCH), which is a repressor of the membrane protein smoothened (SMO). After that, SMO-free interacts with the EVC complex (Ellis van Creveld Syndrome) and translocate to the primary cilia. Then, SMO activates glioma-associated oncogene (GLI) family transcription factors and protein kinase A (PKA) to form a macromolecular complex. Finally, the GLI complex passes through the nucleus to stimulate transcription of downstream target genes, resulting in the acceleration of the transition of telogen to the anagen stage, and enhancing hair follicle development by promoting the proliferation of HFDPCs, mesenchymal, epithelial, and fibroblast cells in the anagen phase [8,61].

Furthermore, vascular endothelial growth factor (VEGF) plays a vital role in promoting angiogenesis during the growth stage of hair follicles. Its function involves enhancing oxygen and nutrient delivery to the follicles, leading to an increase in follicle diameter and stimulating hair growth [62]. The effects of the Hair Rise<sup>TM</sup> microemulsion on genes such as *CTNNB1*, *SHH*, *SMO*, *GLI1*, and *VEGF*, which are involved in hair growth pathways, were evaluated. In HFDPCs, the Hair Rise<sup>TM</sup> microemulsion and standard controls, including minoxidil and purmorphamine, were determined at a concentration of 0.5 mg/mL.



**Figure 4.** The effects of Hair Rise™ microemulsions on the suppression of genes in the androgen pathway; (A) *SRD5A1*, (B) *SRD5A2*, and (C) *SRD5A3* in DU-145 cells; and (D) *SRD5A1*, (E) *SRD5A2*, and (F) *SRD5A3* in HFDPCs were compared to the standard controls (minoxidil, dutasteride, and finasteride) at a concentration of 0.5 mg/mL. HR100: 100% Hair Rise™ microemulsion; HR75: Hair Rise™ microemulsion at a concentration of 75% (*v/v*) in water; HR50: Hair Rise™ microemulsion at a concentration of 50% (*v/v*) in water. The results are shown as a fold change in gene expression relative to the untreated cells (control group). A one-way ANOVA was used for statistical analysis, followed by Tukey's HSD test. Statistical differences ( $p < 0.05$ ) are represented by different letters (a–f).

Considering the stimulation signaling pathway, the Wnt/ $\beta$ -catenin signaling pathway (Figure 5A), and the Sonic Hedgehog signaling pathway (Figure 5B–D), all concentrations of the Hair Rise™ microemulsion significantly stimulated *CTNNB1*, *SHH*, *SMO*, and *GLI1* expression compared to the untreated group and standard controls (minoxidil and purmorphamine) ( $p < 0.05$ ). In the Wnt/ $\beta$ -catenin signaling pathway, the 100% Hair Rise™ microemulsion showed the greatest fold change expression of  $6.86 \pm 0.14$ , followed by the 75% and 50% (*v/v*) Hair Rise™ microemulsion at  $6.42 \pm 0.02$  and  $5.06 \pm 0.02$ , respectively.

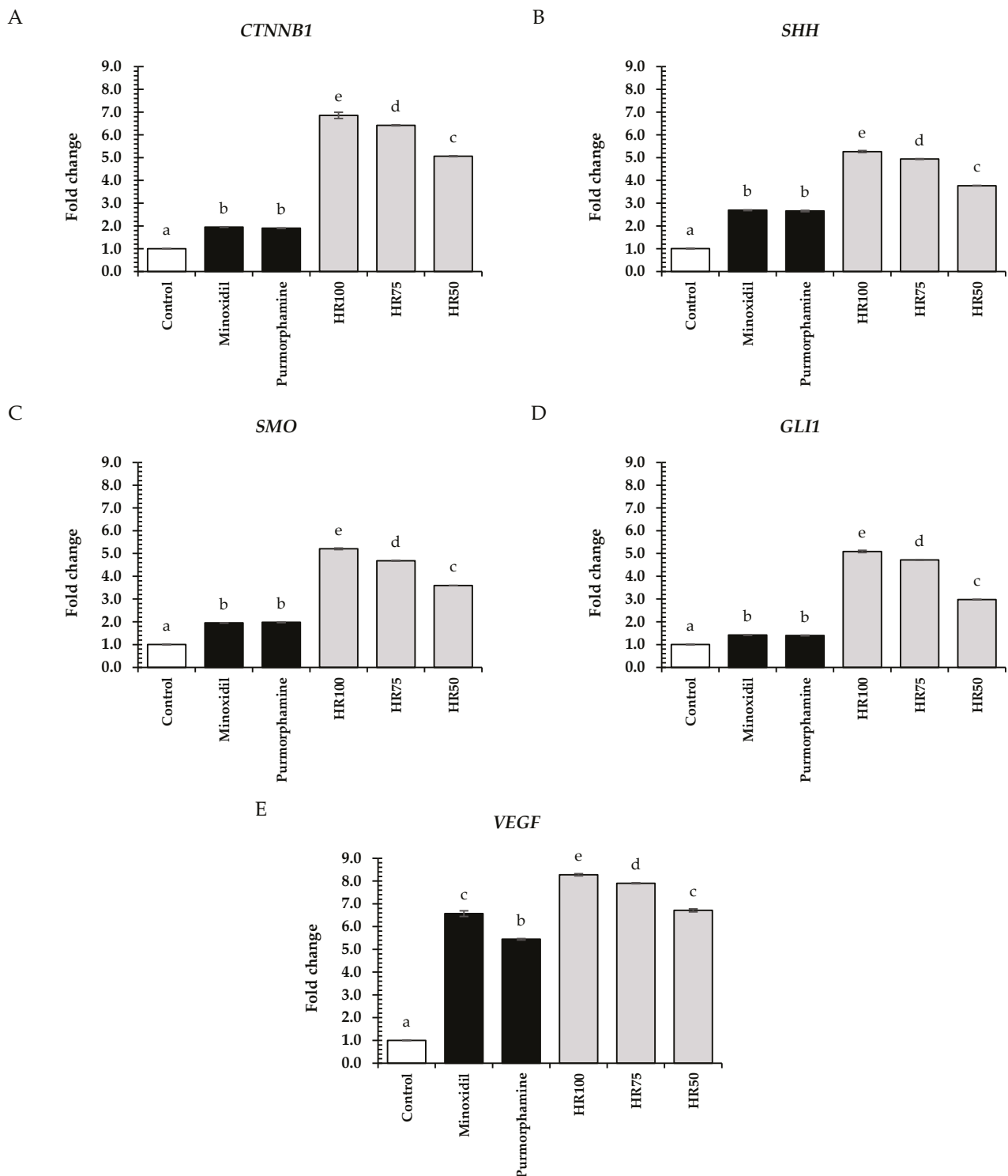
These results suggest that Hair Rise™ microemulsion could stimulate hair follicle growth, which is regulated by Wnt/ $\beta$ -catenin-related genes. For the Sonic Hedgehog signaling pathway, the 100% Hair Rise™ microemulsion expressed the greatest fold change in all tested genes of *SHH* ( $5.26 \pm 0.04$ ), *SMO* ( $5.20 \pm 0.03$ ), and *GLI1* ( $5.08 \pm 0.05$ ), followed by the 75% and 50% (*v/v*) Hair Rise™ microemulsion, respectively. Previous research indicated that chlorogenic acid, epigallocatechin gallate, and quercetin promote the expression of Wnt/ $\beta$ -catenin [16,63,64]. Our results showed that the plant extracts in the Hair Rise™ microemulsion used in this study contain chlorogenic acid, epigallocatechin gallate, and quercetin, which notably increased the accumulation of  $\beta$ -catenin in the cytoplasm of HFDPCs, leading to the transcription of *CTNNB1*. The transcription of *CTNNB1* may support the transition from the telogen to the anagen stage and stimulate hair growth (Table 3) [59]. Furthermore, recent studies have indicated that quercetin in plant extracts promotes hair growth by stimulating the Sonic Hedgehog signaling pathway [65]. Our findings demonstrated that the Hair Rise™ microemulsion, enriched with plant extracts containing quercetin, induced the expression of Sonic Hedgehog-related genes (*SHH*, *SMO*, and *GLI1*) in HFDPCs. These activations may support hair growth by facilitating the transition from the telogen to anagen stage [8]. Additionally, our results demonstrated that the Hair Rise™ microemulsion significantly stimulated the expression of *CTNNB1*, *SHH*, *SMO*, and *GLI1* genes in a concentration-dependent manner.

All concentrations of the Hair Rise™ microemulsion significantly exhibited higher *VEGF* expression activity compared to the untreated cells (control group) and standard controls (minoxidil and purmorphamine) ( $p < 0.05$ ). Especially, the 100% Hair Rise™ microemulsion showed the highest *VEGF* expression ( $8.27 \pm 0.04$ ), followed by the 75% and 50% (*v/v*). This result may be attributed to the combined effects of various compounds in the Hair Rise™ microemulsion, including vitamin B5. Previous research shows that vitamin B5 can stimulate *VEGF* expression and induce wound healing [56]. Furthermore, our results demonstrated that all concentrations of the Hair Rise™ microemulsions showed higher activity than the standard control, especially minoxidil, which is a well-known *VEGF* stimulator that could support angiogenesis during active hair regeneration [11].

**Table 3.** Mechanisms of medicinal plant extracts for hair growth promotion and hair loss prevention.

| Plant Material | Bioactive Compound                                                                                         | Mechanism               | Associated Biomarker                                                                                                                                                            | Reference  |
|----------------|------------------------------------------------------------------------------------------------------------|-------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Rice bran      | Chlorogenic acid<br><i>p</i> -Coumaric acid<br>Rosmarinic acid<br>Ferulic acid<br>Epigallocatechin gallate | Hair growth stimulation | Wnt/ $\beta$ -catenin $\uparrow$<br>Sonic Hedgehog $\uparrow$<br>Angiogenesis $\uparrow$<br>Growth factor ( <i>IGF-1</i> , <i>KGF</i> ) $\uparrow$                              | [2,66]     |
|                | Quercetin<br>$\alpha$ -Tocopherol<br>$\gamma$ -Tocopherol                                                  | Hair loss prevention    | 5 $\alpha$ -reductase $\downarrow$<br>Inflammation $\downarrow$<br>Oxidative stress $\downarrow$                                                                                | [2,66,67]  |
| Shallot bulb   | <i>p</i> -Coumaric acid<br>Rosmarinic acid<br>Quercetin                                                    | Hair growth stimulation | Wnt/ $\beta$ -catenin $\uparrow$<br>Sonic Hedgehog $\uparrow$<br>Angiogenesis $\uparrow$<br>Growth factor ( <i>IGF-1</i> ) $\uparrow$<br>Hair follicle proliferation $\uparrow$ | [16,68]    |
|                |                                                                                                            | Hair loss prevention    | 5 $\alpha$ -reductase $\downarrow$<br>Inflammation $\downarrow$<br>Oxidative stress $\downarrow$                                                                                | [16,69]    |
| Licorice root  | Chlorogenic acid<br>Epigallocatechin gallate<br>Quercetin                                                  | Hair growth stimulation | Hair follicle proliferation $\uparrow$                                                                                                                                          | [21]       |
|                |                                                                                                            | Hair loss prevention    | 5 $\alpha$ -reductase $\downarrow$<br>Inflammation $\downarrow$<br>Oxidative stress $\downarrow$                                                                                | [21,70,71] |
| Corn kernels   | Chlorogenic acid<br><i>p</i> -Coumaric acid<br>Glycyrrhizic acid                                           | Hair loss prevention    | Inflammation $\downarrow$<br>Oxidative stress $\downarrow$                                                                                                                      | [72,73]    |

Note: IGF-1: insulin-like growth factor-1; KGF: keratinocyte growth factor.



**Figure 5.** Effects of Hair Rise™ microemulsions and standard control (minoxidil and purmorphamine) (0.5 mg/mL) on the relative mRNA expression of genes associated with Wnt/ $\beta$ -catenin signaling (A) *CTNNB1*; Sonic Hedgehog pathways (B) *SHH*; (C) *SMO*; (D) *GLI1*; and angiogenesis (E) *VEGF* in HFDPs. HR100: 100% Hair Rise™ microemulsion; HR75: Hair Rise™ microemulsion at a concentration of 75% (v/v) in water; HR50: Hair Rise™ microemulsion at a concentration of 50% (v/v) in water. The results are shown as a fold change in gene expression relative to the untreated cells (control group). A one-way ANOVA was used for statistical analysis, followed by Tukey's HSD test. Statistical differences ( $p < 0.05$ ) are represented by different letters (a–e).

### 3. Materials and Methods

#### 3.1. Chemicals and Reagents

PEG-40 hydrogenated castor oil, Tween 20, Tween 80, and Propylene glycol were purchased from NSG Namsiang (Namsiang Co., Ltd., Chiang Mai, Thailand), while the following substances were obtained from MySkinRecipes (Chanjao Longevity Co., Ltd., Bangkok, Thailand): vitamin B5, disodium EDTA, and sodium hydroxide. The Folin–Ciocalteu reagent, Triton X-100, aluminum chloride hexahydrate, and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). The 2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), epigallocatechin gallate (EGCG), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sulforhodamine B (SRB), diclofenac sodium, L-ascorbic acid, and dimethyl sulfoxide (DMSO) were sourced from Sigma Chemical (St. Louis, MO, USA). Thiobarbituric acid was obtained from VWR Chemicals (BDH Chem. Ltd., Poole, UK). Furthermore, finasteride, dutasteride, and minoxidil were sourced from Wuhan W&Z Biotech (Wuhan, China). For cell culture, the Dermal Papilla Growth Medium was purchased from Promo Cell GmbH (Heidelberg, Germany), while Roswell Park Memorial Institute 1640 Medium (RPMI-1640), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), antibiotic-antimycotic (100×), and penicillin/streptomycin (100×) solutions were sourced from Gibco Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Griess reaction colorimetric kit was sourced from Invitrogen (Thermo Fisher Scientific, Inc., Eugene, OR, USA). All other chemicals used in this study were of analytical grade.

#### 3.2. Plant Materials and Preparation of Extract

The bran of *Oryza sativa* cv. Bue Bang 3-CMU (BB3-CMU) was acquired from the Lanna Rice Research Center, Chiang Mai University, Chiang Mai, Thailand, from June to August 2021. The shallot bulb (*Allium ascalonicum* L.), licorice root (*Glycyrrhiza glabra* L.), and corn kernels (*Zea mays* L.) were purchased from a regular market in Chiang Mai, Thailand, between October and December 2021. Each plant was identified by the Pharmaceutical and Natural Products Research and Development Unit (PNPRDU), Chiang Mai University, Chiang Mai, Thailand, with the code numbers PNPRDU65007, PNPRDU65008, PNPRDU65009, and PNPRDU65010, respectively. Supercritical carbon dioxide (ScCO<sub>2</sub>) was used to extract the BB3-CMU rice bran and shallot bulb according to our previous study. In short, the samples were mixed with a co-solvent and added into the extraction column of the ScCO<sub>2</sub> machine (Applied Separations, Allentown, PA, USA) [28]. At the same time, the corn kernels and licorice root were macerated (CE). The extraction conditions of each plant, including the type of co-solvents, the ratios between plant material and solvent, pressure, temperature, and time, are shown in Table 4. After the extraction, the extract solution was filtered through Whatman filter paper no. 1 and evaporated at 50 °C by an evaporator until completely dried [32,74]. Moreover, guava (*Psidium guajava* L.) extract and the bran extracts of *Oryza sativa* cv. Bue Bang 4-CMU (BB4-CMU), KDML105, and Pieisu 1-CMU were derived from our previous study [2,17,75].

**Table 4.** Extraction methods and the extraction conditions of plant material extracts.

| Plants Materials  | Extraction Method | Co-Solvent           | Ratio (Sample: Co-Solvent) | Pressure (Bar) | Temperature (°C) | Time (h) |
|-------------------|-------------------|----------------------|----------------------------|----------------|------------------|----------|
| BB3-CMU rice bran | ScCO <sub>2</sub> | 95% (v/v)<br>Ethanol | 1:1                        | 400            | 50               | 0.5      |
| Shallot bulb      | ScCO <sub>2</sub> | 50% (v/v)<br>Ethanol | 2:1                        | 400            | 50               | 0.5      |
| Licorice root     | CE                | 30% (v/v)<br>Ethanol | 1:2                        | -              | 25               | 24       |
| Corn kernels      | CE                | 50% (v/v)<br>Ethanol | 1:2                        | -              | 25               | 24       |



### 3.3. Phytochemical Evaluations of Plant Extract

#### 3.3.1. Total Phenolic Content

The total phenolic content of plant extract was measured following the method described by Ruksiriwanich et al., with minor adjustments. In summary, the extract was reacted with 10% (*v/v*) Folin–Ciocalteu reagent in a microtiter plate. Afterward, 6% (*w/v*) sodium bicarbonate solution was added and incubated for 2 h. The absorbance was recorded at 765 nm using a UV–Vis spectrophotometer (EZ Read 400 Flexi, Biochrom, Cambridge, UK) [28]. Gallic acid (10–200 mg/mL) served as the standard, and results were reported as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract). All experiments were conducted in triplicate.

#### 3.3.2. Total Flavonoid Content

The total flavonoid content was measured following the method described by Ruksiriwanich et al., with minor adjustments [16]. Briefly, the plant extract was mixed with distilled water and 7.5  $\mu$ L of a 5.0% (*w/v*) NaNO<sub>2</sub> solution, respectively. The mixture solution was reacted for 5 min. Afterward, 10% (*w/v*) AlCl<sub>3</sub>·6H<sub>2</sub>O solution, 1 M NaOH, and distilled water were loaded into a mixture solution, respectively. Lastly, the absorbance was measured at 510 nm using a UV–Vis spectrophotometer. Epigallocatechin gallate (30–300 mg/mL) served as the standard, and results were reported as a milligram of epigallocatechin gallate equivalents per gram of extract (mg EGCG/g extract). All experiments were conducted in triplicate.

#### 3.3.3. Quantitative Analysis of Glycyrrhizic Acid, Phenolic, and Flavonoid Profiles by Liquid Chromatography–Quadrupole Time-of-Flight Mass Spectrometry

The phenolic, flavonoid, and glycyrrhizic acid of the plant extracts were characterized using LC–QTOF–MS, adapted from a previously used method [76]. The analysis was conducted using an Agilent 1290 Infinity II system coupled with a 6546 LC–QTOF instrument (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved on a ZORBAX Eclipse Plus C18 column (2.1  $\times$  150 mm, 1.8  $\mu$ m) over a 26 min run. The mobile phase comprised solvent A (acetonitrile) and solvent B (distilled water with 0.1% formic acid). A gradient elution started at 50% solvent A for 10 min, gradually increasing to 100% solvent B over 15 min, and held until the end of the run. The flow rate was maintained at 1000  $\mu$ L/min with an injection volume of 10  $\mu$ L. The instrument operated in positive electrospray ionization (ESI) mode, with the following settings: capillary voltage of 4500 V, nebulizer gas (N<sub>2</sub>) at 35 psi, flow rate of 8000 mL/min, and a dry heater temperature of 320  $^{\circ}$ C. Mass spectra were acquired in the *m/z* range of 50–1000. Compound identification was facilitated using the ChemSpider online database.

#### 3.3.4. Quantitative Analysis of $\gamma$ -Oryzanol and Tocopherol by High Performance Liquid Chromatography (HPLC)

The analysis of  $\gamma$ -oryzanol and tocopherol was adapted from the method of Pestana-Bauer et al. [77]. For  $\gamma$ -oryzanol quantification, plant extracts were diluted in dichloromethane to a final concentration of 1000  $\mu$ g/mL, then filtered through a 0.45  $\mu$ m syringe filter. The plant extracts were analyzed using an HPLC machine (Shimadzu, Kyoto, Japan) with an Ultra C18 column (5  $\mu$ m, 4.6  $\times$  250 mm; Restek, PA, USA) and a Shimadzu UV–Vis detector coupled with an SPD-20A diode array detector. The mobile phase consisted of methanol, acetonitrile, dichloromethane, and acetic acid in a ratio of 50:44:3:3, with a flow rate of 1400 mL/min, and the detection wavelength was set at 330 nm.

For tocopherol analysis, a Shimadzu HPLC machine equipped with a fluorescence detector (RF-20A; Shimadzu Corporation, Kyoto, Japan) was used. The separation was carried out on a reverse-phase Ultra C18 column (5  $\mu$ m, 250  $\times$  4.6 mm). The mobile phase consisted of three mixtures: acetonitrile, methanol, and isopropanol in the ratios 50:40:10 (A) and 30:65:5 (B). The gradient started with 85% A for 15 min, decreased to 10% A over 2 min, then increased back to 50% over 5 min, and finally to 85% over 3 min. The flow rate was set

at 1000 µL/min, with a total run time of 26 min. The fluorescence detector was set to an excitation wavelength of 290 nm and an emission wavelength of 330 nm.

### 3.3.5. Determination of Antioxidant Activities

#### DPPH Radical Scavenging Assay

The DPPH radical-scavenging activity was evaluated as previously described [28]. In brief, the plant extract was loaded into a microtiter plate, followed by distilled water and a 0.1 mM ethanolic DPPH solution. After that, the plate was stored in the dark for 30 min. The absorbance of the mixture solution was read at a wavelength of 515 nm using the UV–Vis spectrophotometer. Trolox was employed as a standard scavenger, and 95% (v/v) ethanol was used as a blank in these experiments. The DPPH radical scavenging activity was calculated using Equation (1):

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\text{Optical density}_{\text{control}} - \text{Optical density}_{\text{sample}})}{\text{Optical density}_{\text{control}}} \times 100 \quad (1)$$

where  $\text{Optical density}_{\text{control}}$  = absorbance of the DPPH solution mixture and ethanol, and  $\text{Optical density}_{\text{sample}}$  = absorbance of the DPPH radical solution mixture and sample.

#### ABTS Radical Scavenging Assay

The ABTS assay was performed as previously described [2]. In short, the ABTS stock solution was prepared in equal amounts of two stock solutions (7.0 mM ABTS solution and 2.45 mM potassium persulfate solution). The mixture was stored in the dark for 12–16 h to react. After that, the ABTS stock solution was diluted with 95% (v/v) ethanol to acquire an absorbance of  $0.70 \pm 0.02$  units 734 nm using a UV–Vis spectrophotometer. The ABTS working solution was prepared daily. Then, the plant extract and ABTS working solution were loaded into a microtiter plate and kept in the dark for 30 min. The absorbance of the mixture solution was recorded at 734 nm. Trolox was used as the standard, and 95% (v/v) ethanol served as the blank. The ABTS radical scavenging activity was calculated using Equation (2):

$$\text{ABTS radical scavenging activity (\%)} = \frac{(\text{Optical density}_{\text{control}} - \text{Optical density}_{\text{sample}})}{\text{Optical density}_{\text{control}}} \times 100 \quad (2)$$

where  $\text{Optical density}_{\text{control}}$  = absorbance of the ABTS solution mixture and ethanol, and  $\text{Optical density}_{\text{sample}}$  = absorbance of the ABTS radical solution mixture and sample.

### 3.4. Preparation of Hair Rise™ Microemulsion

A Hair Rise™ microemulsion for hair growth promotion and anti-hair loss was prepared in three phases (A–C). First, the ingredients of the oil based phase (A), including BB3-CMU rice bran extract, BB4-CMU rice bran extract [17], KDML105 rice bran extract [2], Piesu rice bran extract [75], shallot extract [16], and licorice root extract, were weighted and dissolved in Tween 80, Tween 20, and PEG-40 hydrogenated castor oil as co-solvents. For the water-based phase (B), vitamin B5, guava extract [24], and disodium EDTA were dissolved in distilled water. Finally, for the propylene glycol-based phase (C), corn kernel extract was dissolved in propylene glycol.

For the preparation of Hair Rise™ microemulsion according to the drug delivery system (low energy), phase A was heated in a magnetic stirrer set to 40 °C at 300 rpm. Phase B was added to the A phase, maintaining continuous stirring for 10 min. After that, the C phase was added and mixed until fully dissolved. The prepared Hair Rise™ microemulsion was removed from the hot plate magnetic stirrer and transferred to a container for cooling. The composition of the Hair Rise™ microemulsion is shown in Table 5.

**Table 5.** Compositions of Hair Rise™ microemulsion (*w/w*).

| Phase | Ingredient                     | Quantity % ( <i>w/w</i> ) |
|-------|--------------------------------|---------------------------|
| A     | BB3-CMU rice bran extract      | 1                         |
|       | BB4-CMU rice bran extract      | 1                         |
|       | KDML105 rice bran extract      | 1                         |
|       | Piesu 1 CMU rice bran extract  | 1                         |
|       | Tween 80                       | 8.5                       |
|       | Tween 20                       | 2                         |
|       | PEG-40 hydrogenated castor oil | 10                        |
| B     | Vitamin B5                     | 1                         |
|       | Guava extract                  | 2                         |
|       | Shallot extract                | 1                         |
|       | Licorice root extract          | 1.5                       |
|       | Disodium EDTA                  | 0.5                       |
|       | Distilled water                | 56.5                      |
| C     | Corn kernels extract           | 3                         |
|       | Propylene glycol               | 10                        |

### 3.5. Measurement of Particle Size, Polydispersity Index, and Zeta Potential Values of Hair Rise™ Formulation

The particle size, or Z-average (Z-Ave), polydispersity index (pdl), and zeta potential (ZP) value of Hair Rise™ microemulsion were determined using Zetasizer Nano ZS with a dynamic light scattering technique (Malvern Panalytical, Bristol, UK). The physical stability of Hair Rise™ microemulsion was evaluated by measuring Z-Ave, pdl, and ZP after storing the microemulsion at 30 °C for 3 months, with assessments at initial, 1, 2, and 3 months. The heating–cooling cycle test was conducted to assess the stability of Hair Rise™ microemulsion under extreme temperature variations, such as during transportation. In this test, the microemulsion was alternately stored at 4 °C and 45 °C for 24 hours per temperature (one cycle), and this process was repeated for 6 cycles. All of the samples were evaluated using integrated dynamic light scattering (DLS) for 1 min. The Malvern’s Dispersion Technology Software (DTS) version 7.13 was used to calculate the DLS data [78].

### 3.6. In Vitro Cell Viability Assay

The primary human hair follicle dermal papilla cells (HFDPCs) were obtained from Promo Cell GmbH (Heidelberg, Germany) and cultured using the Dermal Papilla Growth Medium, supplemented with a 1% antibiotic-antimycotic solution. Furthermore, human prostate cancer cells (DU-145) and macrophage cells (RAW 264.7) were sourced from the American Type Culture Collection (Rockville, MD, USA). DU-145 cells were maintained in RPMI-1640 culture medium with the addition of 10% FBS and 1% penicillin/streptomycin solution. RAW 264.7 cells were maintained in DMEM culture medium, also supplemented with 10% FBS and 1% penicillin/streptomycin solution.

The sulphorhodamine B (SRB) method was used to assess the viability and proliferation of cells treated with plant extracts, Hair Rise™ microemulsion at varying concentrations (50, 75, and 100% *v/v* in water), and standard controls (including diclofenac sodium, dutasteride, finasteride, and minoxidil) at concentrations ranging from 0.0625 to 2 mg/mL in RAW 264.7, DU-145, and HFDPCs [79]. Cells were added into 96-well plates at a density of  $1 \times 10^5$  cells/mL and incubated for 24 h at 37 °C with 5% CO<sub>2</sub>. Then, the cells were treated with the samples and exposed to all treatments for 24 h. Afterward, the monolayer cells were fixed with 50% (*w/v*) trichloroacetic acid at 4 °C for 1 h, followed by staining with 0.04% (*w/v*) SRB solution for 30 min. Lastly, the bound dye was removed by 10 mM Tris base, and the absorbance was read at 515 nm. The concentrations that provided more

than 80% cell viability were collected for further experiments. Cell viability was calculated using Equation (3).

$$\text{Cell viability (\%)} = \frac{(\text{Optical density}_{\text{sample}} - \text{Optical density}_{\text{blank}})}{(\text{Optical density}_{\text{control}} - \text{Optical density}_{\text{blank}})} \times 100 \quad (3)$$

where  $\text{Optical density}_{\text{sample}}$  is the absorbance of the cells treated with plant extracts, Hair Rise™ microemulsions or the standard controls,  $\text{Optical density}_{\text{control}}$  is the absorbance of the cells treated with ethanol in culture medium, and  $\text{Optical density}_{\text{blank}}$  is the absorbance of the 96-well plate.

### 3.7. Anti-Inflammatory Activity Assay

The quantification of nitrite that increased in the cell culture supernatants was evaluated by the Griess reaction colorimetric assay kit. In short, the HFDPCs and RAW 264.7 cells were seeded ( $1 \times 10^5$  cells/mL) and incubated for 24 h. Afterward, the cells were pretreated with 0.5 mg/mL of standard controls (diclofenac sodium), Hair Rise™ microemulsion in different concentrations (50, 75, and 100% (v/v) in water), and incomplete medium (blank) for 2 h. Afterward, the cells were exposed to 0.001 mg/mL LPS for 24 h. Cell culture supernatants were collected and reacted with the Griess reagent according to the manufacturer's instructions. A calibration curve was generated from standard nitrite at a concentration of 0.01 to 100 µM [76].

### 3.8. Thiobarbituric Acid-Reactive Substances (TBARS) Assay

The antioxidant activity of standard controls (L-ascorbic acid), Hair Rise™ microemulsion in different concentrations (50, 75, and 100% (v/v) in water), and the control group (incomplete medium) was determined using the TBARS method. In brief, HFDPCs were added to 6-well plates ( $1 \times 10^5$  cells/mL) and incubated for 24 h. Subsequently, the cells were pretreated with 0.5 mg/mL of each sample for 24 h, followed by post-treatment with  $\text{H}_2\text{O}_2$  for 2 h. The cells were harvested and reacted with a mixture solution (1% Triton X-100, 0.6% thiobarbituric acid, and 15% trichloroacetic acid) at 100 °C for 10 min. Afterward, the cells were cooled down in a −80 °C freezer for 10 min. The final product of lipid peroxidation was read at 532 nm. The quantification of malondialdehyde in HFDPCs was determined in comparison to the untreated cells (control group) [76].

### 3.9. Semi-Quantitative Reverse Transcription and Polymerase Chain Reaction Analysis

The expression of the gene in the androgen pathway (*SRD5A1-3*) and the genes associated with hair growth, including Wnt/β-catenin (*CTNNB1*), Sonic Hedgehog (*SHH*, *SMO*, and *GLI1*), and angiogenesis pathways (*VEGF*), was evaluated in DU-145 and HFDPCs as previously established [28]. Hair Rise™ microemulsion at different concentrations (50, 75, and 100% (v/v) in water) was compared to the standard controls (dutasteride, finasteride, and minoxidil) at 0.5 mg/mL. RNA was extracted using the E.Z.N.A.® Total RNA Kit I (Omega BioTek, Norcross, GA, USA). The RNA concentration was quantified utilizing the Qubit™ RNA HS Assay Kit alongside the Qubit™ 4 fluorometer (Invitrogen, Carlsbad, CA, USA). Gene expression analysis was carried out with the MyTaq™ One-Step RT-PCR Kit (Bioline, Memphis, TN, USA), and the specific primer sequences used are listed in Table 6. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. The results were shown as fold changes in gene expression. PCR products were visualized by agarose gel electrophoresis, with images and band intensity analyzed using the Gel Doc™ EZ System and Image Lab™ software 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

**Table 6.** Specific primer sequences used for semi-quantitative RT-PCR.

| Functional Pathway    | Gene Name     | Gene Bank No.  | Primers | Sequence (5'-3')       |
|-----------------------|---------------|----------------|---------|------------------------|
| Androgen              | <i>SRD5A1</i> | NM_001047.4    | Forward | AGCCATTGTGCAGTGTATGC   |
|                       |               |                | Reverse | AGCCTCCCCTTGGTATTTTG   |
|                       | <i>SRD5A2</i> | NM_000348.4    | Forward | TGAATACCCTGATGGGTGG    |
|                       |               |                | Reverse | CAAGCCACCTTGTGGAATC    |
|                       | <i>SRD5A3</i> | NM_024592.5    | Forward | TCCTTCTTTGCCCCAACATC   |
|                       |               |                | Reverse | TCCTTCTTTGCCCCAACATC   |
| Wnt/ $\beta$ -catenin | <i>CTNNB1</i> | NM_001330729.2 | Forward | CCCACTAATGTCCAGCGTTT   |
|                       |               |                | Reverse | AACCAAGCATTTTCACCAGG   |
| Sonic Hedgehog        | <i>SHH</i>    | NM_000193.4    | Forward | AAAAGCTGACCCCTTTAGCC   |
|                       |               |                | Reverse | GCTCCGGTGTTTTCTTCATC   |
|                       | <i>SMO</i>    | NM_005631.5    | Forward | GAAGTGCCCTTGGTTCGGACA  |
|                       |               |                | Reverse | CCGCCAGTCAGCCACGAAT    |
|                       | <i>GLI1</i>   | NM_005269.3    | Forward | GCAGGGAGTGCAGCCAATACAG |
|                       |               |                | Reverse | GAGCGGCGGCTGACAGTATA   |
| Angiogenesis          | <i>VEGF</i>   | NM_001025366.3 | Forward | CTACCTCCACCATGCCAAGT   |
|                       |               |                | Reverse | GCGAGTCTGTGTTTTTGCAG   |
| Internal control      | <i>GAPDH</i>  | NM_001289745.3 | Forward | GGAAGGTGAAGGTCGGAGTC   |
|                       |               |                | Reverse | CTCAGCCTTGACGGTGCCATG  |

### 3.10. Statistical Analysis

The results are expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were performed using one-way ANOVA, followed by Tukey's post hoc test, with significance defined at  $p < 0.05$ . Data processing and analysis were conducted using statistical software (version 23.0).

## 4. Conclusions

A green cosmeceutical Hair Rise<sup>TM</sup> microemulsion containing medicinal extracts from ScCO<sub>2</sub> BB3-CMU rice bran, BB4-CMU rice bran, KDML105 rice bran, Piesu 1 CMU rice bran, guava leaf, shallot bulb, licorice root, and corn kernels was successfully formulated to promote hair growth and combat hair loss. Hair Rise<sup>TM</sup> microemulsion at all concentrations (50%, 75%, and 100% *v/v* in water) promoted cell proliferation of HFDPCs, particularly during the anagen phase of the hair cycle. They also demonstrated anti-inflammatory and antioxidative properties by suppressing nitric oxide and TBARS levels, which reduced inflammation and oxidative damage in hair follicles. In addition, all concentrations of Hair Rise<sup>TM</sup> microemulsion showed significantly higher inhibitory effects on *SRD5A* expression than minoxidil, finasteride, and dutasteride, which could decrease the synthesis of androgenic hormone in hair follicles and present an anti-hair loss effect. Furthermore, all concentrations of Hair Rise<sup>TM</sup> microemulsion stimulated the expression of hair growth factor genes, including *CTNNB1*, *SHH*, *SMO*, *GLI1*, and *VEGF*, to support hair growth by extending the anagen phase and enhancing blood flow to the hair follicles. In summary, our findings suggest that Hair Rise<sup>TM</sup> microemulsion at all concentrations, particularly the 100% formulation, represents the most effective treatment for AGA, highlighting the potential of green cosmetics in promoting hair growth and minimizing hair loss. However, a further efficacy test of Hair Rise<sup>TM</sup> microemulsion should be arranged in AGA patients.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants13192802/s1>, Table S1: The effects of different proportions of plant extracts on the gene expression of *SRD5A2* in DU-145 cells.



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**Data Availability Statement:** Data are contained within the article and Supplementary Materials.

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## Article

# Effects of Micronutrients on the Growth and Phytochemical Composition of Basil (*Ocimum basilicum* L.) in the Field and Greenhouse (Hydroponics and Soil Culture)

Hamid Aghamirzaei <sup>1</sup>, Hasan Mumivand <sup>1,\*</sup>, Abdollah Ehtesham Nia <sup>1</sup>, Mohamad Reza Raji <sup>1</sup>, Alfred Maroyi <sup>2</sup> and Filippo Maggi <sup>3</sup>

<sup>1</sup> Department of Horticultural Sciences, Faculty of Agriculture, Lorestan University, Khorramabad P.O. Box 465, Iran

<sup>2</sup> Department of Botany, University of Fort Hare, Alice 5700, South Africa

<sup>3</sup> Chemistry Interdisciplinary Project (ChIP) Research Center, School of Pharmacy, University of Camerino, 62032 Camerino, Italy

\* Correspondence: mumivand.h@lu.ac.ir or hmumivand@gmail.com

**Abstract:** The current research was conducted to compare the growth, yield, and phytochemical composition of basil (*Ocimum basilicum*) in the open field and the soil and hydroponic cultivation in a greenhouse. Furthermore, the effect of foliar spraying of micronutrients on this crop was also evaluated. In each of the cultivation systems, foliar spraying of one micronutrient, either iron sulfate (Fe), zinc sulfate (Zn), copper sulfate (Cu), manganese sulfate (Mn), or boric acid (B), at a concentration of 0.1% was applied in a randomized complete block design. Plants grown in the hydroponic system had higher yield and biomass. The concentration of the elements K, Ca, Mg, N, P, Mn, Fe, B, and Zn in the leaves of hydroponic plants was higher. Contrarily, plants cultivated in the field showed higher stem dry weight, essential oil content, phenolic and flavonoid content, and antioxidant activity. The level of methyl chavicol was higher in the hydroponic culture, but the level of 1,8-cineole was much lower in this cropping system. Foliar spraying of Cu, Mn, Zn, Fe, or B significantly increased leaf dry weight and anthocyanin content. In field conditions, the highest levels of phenolics, flavonoids, and antioxidant capacity were observed with Zn or Mn application. In the hydroponic system, foliar spraying of Zn or B led to the highest antioxidant capacity, and total phenolic and flavonoid contents. Overall, the basil plants cultivated in the field showed higher bioactive ingredients. However, the essential oil of plants cultivated in the hydroponic system had a higher economic value due to its higher percentage of methyl chavicol.

**Keywords:** essential oil; phenol; flavonoid; antioxidant activity

## 1. Introduction

Basil (*Ocimum basilicum* L.) is an important member of the Lamiaceae family, widely used as a medicinal plant, spice, and fresh vegetable. The bioactive ingredients of this plant are used to treat flatulence and anorexia and aid digestion [1]. Moreover, its extract and essential oil are widely used in the food industry as a flavoring agent [2]. Phenolics in vegetables and plants are the main bioactive compounds known for their antioxidant properties [3]. Basil essential oil contains monoterpenoids (limonene, camphor, linalool, geraniol) and phenylpropanoids (eugenol, methyl chavicol), which are also responsible for the medicinal properties of this plant [4]. Essential oils are complex mixtures of volatile compounds, whose quantity and quality are affected by various factors such as genetics, climatic and geographical conditions (elevation of habitat, quantity and quality of light, temperatures, availability of water, salinity, soil characteristics), and agricultural operations (access to macroelements and microelements, irrigation regimes, tillage) [5–7].



There are different types of cultivation systems, which differ from each other in terms of ideology or technology. In recent years, due to the shortages of water or the salinity of water and soil in many regions of the world, the focus has been directed to the production of high-quality horticultural crops under controlled conditions, and new alternative techniques have been developed to produce these plants at a high density [8]. Outdoor agriculture faces many challenges, including soil-borne diseases, lack or inadequate levels of organic matter in the soil, infeasibility of cultivating certain products, lack of precise control of nutrients at different stages of plant growth in the soil, difficulty of weed control, low efficiency of water consumption, and impossibility of controlling environmental conditions. Therefore, despite the availability of soil resources and land suitable for agriculture in some areas, due to the above challenges, it is necessary to use alternative methods to produce crops. A suitable method to achieve high agricultural production in small areas is to use controlled environment agriculture so that while increasing the production per unit area, high-quality crops in the desired quantities can be produced throughout the year [9,10]. The increasing interest in soilless cultivation is due to the many problems associated with the open field cultivation, such as soil diseases, weeds, and issues related to rotation [11,12]. In addition, the lack of water and the impossibility of precisely controlling plant nutrition in soil systems have led to an increase in the production of crops through different methods of soilless cultivation in recent decades [13,14]. Some of the advantages of hydroponic cultivation are the lack of contamination with soil diseases, higher crop yields, no dependence on soil quality, and the ability to precisely control water and nutrients [9,13].

Comparing the yield and quality of crops in different cropping systems is often challenging due to the intricate interplay between genotype, plant physiology, and the environment. Nevertheless, it is crucial to carry out controlled comparisons in order to pinpoint the most efficient and effective food production systems [10]. Maliqa et al. [12] stated that in the hydroponic cultivation system, the rate of photosynthesis and plant yield were significantly higher than in the soil-based system. However, a significant decrease in dry matter was observed in hydroponic conditions. A comparison of hydroponic and field cultivation of *Carlina acaulis* L. showed that the antioxidant capacity and content of carlina oxide were significantly higher in field plants. However, the flavonoid content in the leaves of plants grown in both systems was similar [15]. In the comparison of tissue culture plants, field culture, and hydroponic culture of *Acmella oleracea* (L.) R.K.Jansen, the content of total phenolics, total flavonoids, and antioxidants in plants from hydroponic culture was higher than in field-grown plants and in callus from tissue culture [14].

The concentration of macroelements and microelements in the soil is one of the key factors in the production and accumulation of bioactive substances in medicinal plants, and the level of their absorption has a great effect on the biosynthesis of medicinal compounds [16,17]. Among the micronutrients, Zn plays a vital role in cellular functions such as indole acetic acid (IAA) synthesis, protein metabolism, and photosynthesis, and its deficiency causes thickening of leaves, premature loss of foliage, and stunted growth. Fe plays a vital role in chlorophyll synthesis, carbohydrate production, and cellular respiration. Fe deficiency results in interveinal chlorosis of younger leaves, commonly known as iron chlorosis [18]. B plays a crucial role in stabilizing certain components of the cell wall and plasma membrane; it contributes to the strengthening of cell division and supports the metabolism of nucleic acids, carbohydrates, proteins, auxins, and phenolics. Generally, plants are prone to Zn, Fe, and B deficiency in alkaline soil with a coarse texture and low organic matter. The deficiency of Zn, Fe, and B (or their insufficient fertilization) may be a significant reason for low productivity [6,19]. In calcareous soils, Cu, Fe, Mn, and Zn are less available because they precipitate in the soil to form carbonates or bicarbonates, and they are also less available in soils with high organic matter [20]. Micronutrients such as Fe, Cu, Mn, and Zn are key factors for the activity of various enzymes in plants, especially those affecting the biosynthesis of secondary metabolites [17].

Inadequate availability of organic matter in the soil, high salinity, low humidity, high bicarbonate ions in irrigation water, high lime and soil pH, and imbalanced use of fertilizers

lead to deficiencies of micronutrients in plants. Deficiency of nutrients is common in almost all farms in the world and its level varies in different regions and from plant to plant [21]. Foliar spraying of nutrients is a common method of meeting the nutritional demands of higher plants, which is more efficient than soil application in poor soils in terms of nutrient availability [6,22]. Spraying basil with Zn chelates under salinity stress conditions improved the growth and yield of the plant, increased the percentage and yield of essential oil, and raised the levels of linalool and methyl chavicol, the dominant components of basil's essential oil [23]. The application of Zn (50  $\mu$ M) increased the concentration of polyphenols, carotenoids, and antioxidant capacity of the two basil varieties by 14.57, 19.76, and 33.72%, respectively, compared to the control [24]. B or Zn foliar application improved the content of total phenolics, essential oil content and yield, and antioxidant capacity of *Satureja khuzistanica* Jamzad. Furthermore, the application of B increased the production of carvacrol in the essential oil [6].

In addition to having an essential role in the growth and yield of medicinal plants and vegetables, micronutrients are effective in increasing their quality and nutritional value. Considering the importance of basil as a fresh vegetable and its special use in the nutraceutical industry, there is a high demand for consuming this vegetable throughout the year. Therefore, to address the requirements of the growing human population, there is a need to develop new agricultural systems. In addition, growth rates, yields, and crop quality vary across modern cultivation systems. A review of the literature also shows that no comparison has been made so far between the modern systems of greenhouse cultivation and field cultivation of basil. The primary objective of the present study was to compare the growth, yield, phytochemical characteristics, and nutritional elements of basil cultivated in different environments, specifically in the open field and the soil and hydroponic cultivation in greenhouses. Furthermore, the study aimed to assess the impact of foliar spraying of micronutrient elements on the growth and development of basil plants.

## 2. Results

### 2.1. Yield and Biomass

The analysis of variance (Table S1) showed that the effect of the cultivation system on all parameters related to yield and biomass, including plant height, leaf area, stem dry weight, and leaf dry weight, was significant ( $p \leq 0.01$ ). All traits related to yield and biomass were significantly affected by the foliar spraying of micronutrients. The effect of cultivation system  $\times$  foliar spraying was also significant on plant height, stem dry weight, and leaf area.

The mean comparison of the effect of the cultivation system  $\times$  foliar spraying of micronutrients (Table 1) demonstrated that the highest plant height was found in the hydroponic system with the B, Cu, Mn, and Zn foliar spray treatments ( $60.93 \pm 3.56$ ,  $60.45 \pm 5.32$ ,  $58.81 \pm 3.24$ , and  $58.05 \pm 5.33$  cm, respectively). Contrarily, the lowest plant height was observed in the control treatment and in the field ( $31.07 \pm 1.40$  cm). Overall, plants grown in the hydroponic system were taller than in other systems, and the lowest plant height was obtained in the field cultivation. In field cultivation, the highest plant height was obtained with Fe foliar spray treatment, while in soil and hydroponic greenhouse cultivations, plants treated with B showed the highest plant height. The highest leaf area was obtained in hydroponic cultivation with Mn spraying ( $15.33 \pm 2.42$  cm<sup>2</sup>), which was not significantly different from those in B, Cu, Zn, and Fe treatments in the hydroponic system. The lowest leaf area was observed in the field cultivation with the control treatment ( $5.87 \pm 0.62$  cm<sup>2</sup>). In general, greenhouse-grown plants, especially in the hydroponic system, had a higher leaf area. The highest stem dry weight ( $14.56 \pm 2.22$  g) was found in the field cultivation with Fe foliar application, which did not have a significant difference from those of Cu, Zn, B, or Mn treatment in the field cultivation and Cu, Fe, or Zn treatment in the soil greenhouse cultivation. The lowest stem dry weight was obtained in the soil greenhouse cultivation system with no foliar spray ( $4.25 \pm 0.54$  g), which was not significantly different from the no foliar spray treatment in the hydroponic cultivation

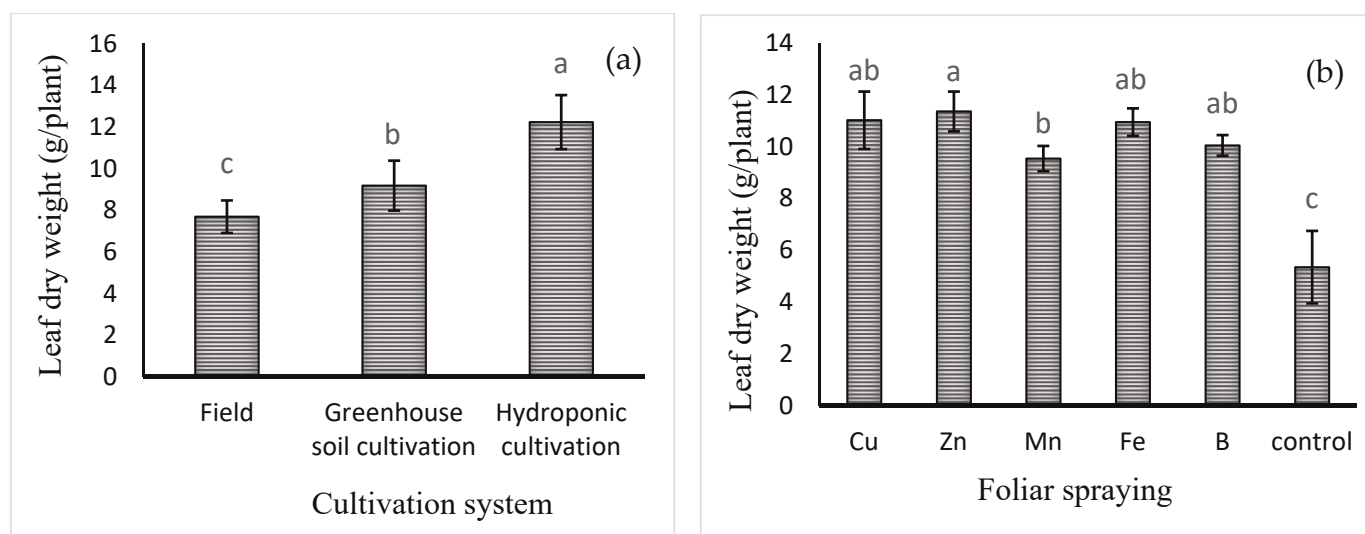
system. The plants grown in the greenhouse showed lower stem dry weight, especially compared to the field cultivation (Table 1).

**Table 1.** Mean comparison of the interaction effect of cultivation system and foliar spraying on biomass and phytochemical characteristics of basil.

| Cultivation System      | Foliar Spray | Plant Height (cm)  | Leaf Area (cm <sup>2</sup> ) | Stem Dry Weight (g) | Essential Oil Yield (g/plant) | Total Phenol (mg GAE/g DW) | Total Flavonoids (mg Q/g DW) | DPPH (IC <sub>50</sub> mg/mL) | FRAP (mmol Fe/g DW) |
|-------------------------|--------------|--------------------|------------------------------|---------------------|-------------------------------|----------------------------|------------------------------|-------------------------------|---------------------|
| Field                   | Control      | 31.07 ± 1.40<br>j  | 5.87 ± 0.62<br>h             | 8.86 ± 0.72<br>cd   | 0.03 ± 0.002<br>ij            | 6.42 ± 1.02<br>de          | 3.61 ± 1.02<br>efg           | 7.57 ± 1.03<br>c              | 3.49 ± 0.42<br>ef   |
|                         | Cu           | 37.84 ± 1.20<br>i  | 9.34 ± 0.43<br>g             | 14.54 ± 1.46<br>a   | 0.06 ± 0.003<br>g-j           | 8.31 ± 1.33<br>cd          | 4.79 ± 1.21<br>de            | 6.46 ± 1.12<br>efd            | 5.60 ± 0.67<br>b    |
|                         | Zn           | 37.44 ± 2.60<br>i  | 9.39 ± 0.72<br>g             | 13.62 ± 1.83<br>a   | 0.30 ± 0.05<br>a              | 16.54 ± 2.42<br>a          | 9.56 ± 2.01<br>a             | 5.41 ± 1.1<br>g               | 7.26 ± 1.02<br>a    |
|                         | Mn           | 40.08 ± 3.46<br>hi | 8.93 ± 1.33<br>g             | 13.86 ± 2.13<br>a   | 0.16 ± 0.04<br>cde            | 12.06 ± 1.91<br>b          | 7.17 ± 1.44<br>b             | 5.84 ± 0.87<br>fg             | 7.15 ± 1.14<br>a    |
|                         | Fe           | 41.87 ± 2.91<br>gh | 9.56 ± 1.33<br>g             | 14.56 ± 2.22<br>a   | 0.14 ± 0.04<br>d-g            | 12.11 ± 2.03<br>b          | 6.58 ± 1.84<br>bc            | 6.63 ± 1.23<br>efd            | 5.41 ± 1.1<br>b     |
|                         | B            | 39.50 ± 4.32<br>hi | 8.90 ± 0.82<br>g             | 14.37 ± 2.33<br>a   | 0.18 ± 0.02<br>cd             | 7.58 ± 1.12<br>cde         | 4.01 ± 1.15<br>d-g           | 5.99 ± 1.42<br>efg            | 7.08 ± 1.13<br>a    |
| Greenhouse soil culture | Control      | 45 ± 4.24<br>fg    | 11.31 ± 1.43<br>f            | 4.25 ± 0.54<br>e    | 0.02 ± 0.004<br>j             | 5.99 ± 1.01<br>e           | 2.45 ± 0.72<br>fgh           | 8.56 ± 1.68<br>b              | 3.94 ± 0.54<br>de   |
|                         | Cu           | 44.46 ± 3.33<br>fg | 12.08 ± 12<br>def            | 12.1 ± 0.95<br>ab   | 0.03 ± 0.006<br>ji            | 8.34 ± 1.35<br>cd          | 5.46 ± 1.44<br>cd            | 6.42 ± 1.51<br>def            | 2.96 ± 0.84<br>fg   |
|                         | Zn           | 44.5 ± 3.65<br>fg  | 13.31 ± 1.31<br>c            | 14.06 ± 1.54<br>a   | 0.13 ± 0.03<br>d-g            | 5.62 ± 0.82<br>e           | 3.80 ± 0.73<br>e-h           | 6.87 ± 1.43<br>cde            | 2.95 ± 0.63<br>fg   |
|                         | Mn           | 47 ± 3.54<br>ef    | 13.84 ± 1.41<br>bc           | 7.87 ± 0.74<br>cd   | 0.10 ± 0.02<br>e-j            | 7.15 ± 1.55<br>cde         | 4.02 ± 1<br>e-h              | 6.76 ± 0.63<br>cde            | 5.36 ± 0.86<br>b    |
|                         | Fe           | 50.33 ± 4.71<br>d  | 12.85 ± 1<br>cde             | 12.05 ± 1.54<br>ab  | 0.06 ± 0.008<br>hi            | 8.98 ± 1.43<br>c           | 4.45 ± 1.32<br>de            | 6.99 ± 1.02<br>cd             | 3.50 ± 0.62<br>ef   |
|                         | B            | 54.66 ± 4.36<br>c  | 11.89 ± 1.31<br>ef           | 9.78 ± 0.91<br>cd   | 0.1 ± 0.04<br>e-i             | 7.02 ± 0.91<br>cde         | 2.32 ± 0.67<br>ghi           | 6.86 ± 1.1<br>cde             | 4.97 ± 0.89<br>bc   |
| Hydroponic culture      | Control      | 49.38 ± 4.22<br>cd | 13.1 ± 1.61<br>cd            | 6.48 ± 0.72<br>de   | 0.03 ± 0.01<br>hij            | 1.57 ± 0.42<br>f           | 0.64 ± 0.12<br>i             | 10.48 ± 2.01<br>a             | 2.17 ± 0.07<br>g    |
|                         | Cu           | 60.45 ± 5.32<br>ab | 14.53 ± 2.31<br>ab           | 8.76 ± 0.82<br>cd   | 0.07 ± 0.02<br>f-j            | 2.40 ± 0.62<br>f           | 0.87 ± 0.05<br>hi            | 10.25 ± 1.7<br>a              | 3.02 ± 0.64<br>fg   |
|                         | Zn           | 58.05 ± 5.33<br>ab | 14.72 ± 2.32<br>ab           | 9.81 ± 1.46<br>bc   | 0.22 ± 0.03<br>cb             | 6.78 ± 1.47<br>cde         | 4.19 ± 1.01<br>def           | 6.95 ± 1.2<br>cd              | 4.36 ± 0.9<br>cde   |
|                         | Mn           | 58.81 ± 3.24<br>ab | 15.33 ± 2.42<br>a            | 8.53 ± 1.16<br>cd   | 0.27 ± 0.05<br>ab             | 2.64 ± 0.18<br>f           | 1.26 ± 0.22<br>hi            | 6.63 ± 1.02<br>def            | 2.95 ± 0.8<br>fg    |
|                         | Fe           | 57.63 ± 3.91<br>b  | 14.51 ± 1.53<br>ab           | 9.37 ± 1.41<br>bcd  | 0.11 ± 0.02<br>e-h            | 5.63 ± 1.02<br>e           | 4.13 ± 0.55<br>def           | 6.55 ± 0.78<br>def            | 2.87 ± 0.64<br>fg   |
|                         | B            | 60.93 ± 3.56<br>a  | 15.13 ± 2.4<br>2a            | 8.47 ± 0.82<br>cd   | 0.15 ± 0.03<br>e-d            | 7.60 ± 1.66<br>cde         | 4.17 ± 1.01<br>def           | 8.52 ± 1.32<br>b              | 4.41 ± 0.88<br>cd   |

Means with the same letters in each column do not have a significant difference at the 1% probability level based on the LSD test.

Mean comparison of the effect of cultivation system on leaf dry weight (Figure 1a) showed that the highest leaf dry weight was observed in hydroponic cultivation ( $12.23 \pm 1.33$  g) and the lowest in field cultivation ( $7.69 \pm 0.78$  g). Furthermore, the mean comparisons (Figure 1b) also showed that the highest leaf dry weight was recorded for Zn foliar spray ( $11.35 \pm 0.77$  g), which was 106% higher compared to the control treatment (no foliar spray), although not significantly different from that of Cu, Fe, or B treatment. The lowest leaf dry weight was obtained in the control treatment ( $5.34 \pm 1.42$  g).



**Figure 1.** Mean comparison of the effects of (a) cultivation system and (b) foliar spraying of micronutrients on basil leaf dry weight. Different lowercase letters indicate significant differences among different treatments.

## 2.2. Macronutrients

The analysis of variance of the effects of cultivation system and foliar spray of micronutrients on the macronutrients of basil is shown in Table S2. The effect of the cultivation system on the concentration of all macronutrients, including N, P, Ca, K, and Mg, was significant. Contrary to this, the simple effect of foliar application of micronutrients and the interactive effect of cultivation system and foliar application of micronutrients were not significant on all of the macronutrients. The mean comparisons of the effect of the cultivation system on the levels of macronutrients in basil leaves are shown in Table 2. The highest levels of all macronutrients, including N ( $4.06 \pm 0.67\%$ ), K ( $1.25 \pm 0.28\%$ ), P ( $0.6 \pm 0.17\%$ ), Mg ( $0.29 \pm 0.03\%$ ), and Ca ( $0.58 \pm 0.06\%$ ), were found in the hydroponic cultivation system, while the lowest were found in the field cultivation system.

**Table 2.** Mean comparison of the effect of the cultivation system on the amount of some macroelements and microelements in basil leaves.

| Cultivation System      | N (%)                | K (%)                | P (%)                | Mg (%)               | Ca (%)               | Cu ( $\mu\text{g/g}$ ) | Zn ( $\mu\text{g/g}$ ) | Fe ( $\mu\text{g/g}$ ) | Mn ( $\mu\text{g/g}$ ) | B ( $\mu\text{g/g}$ ) |
|-------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|------------------------|------------------------|------------------------|------------------------|-----------------------|
| Field                   | $3.36 \pm 0.44$<br>b | $0.60 \pm 0.15$<br>c | $0.36 \pm 0.03$<br>b | $0.22 \pm 0.04$<br>b | $0.35 \pm 0.05$<br>b | $6.50 \pm 0.82$<br>b   | $27.26 \pm 1.76$<br>c  | $58.16 \pm 4.47$<br>c  | $29.74 \pm 3.88$<br>c  | $4.36 \pm 1.12$<br>b  |
| Greenhouse soil culture | $3.41 \pm 0.38$<br>b | $0.62 \pm 0.14$<br>b | $0.52 \pm 0.05$<br>b | $0.23 \pm 0.05$<br>b | $0.37 \pm 0.06$<br>b | $7.94 \pm 1.48$<br>a   | $41.95 \pm 2.45$<br>b  | $64.76 \pm 6.95$<br>b  | $44.18 \pm 6.14$<br>b  | $4.53 \pm 0.88$<br>b  |
| Hydroponic culture      | $4.06 \pm 0.67$<br>a | $1.25 \pm 0.28$<br>a | $0.60 \pm 0.17$<br>a | $0.29 \pm 0.03$<br>a | $0.58 \pm 0.06$<br>a | $6.50 \pm 1.32$<br>b   | $53.36 \pm 3.94$<br>a  | $74.08 \pm 8.75$<br>a  | $94.3 \pm 8.63$<br>a   | $5.66 \pm 1.44$<br>a  |

Means with the same letters in each column do not have a significant difference at the 1% probability level based on the LSD test.

## 2.3. Micronutrients

The analysis of variance (Table S2) revealed that the effects of the cultivation system and foliar spraying of micronutrients were significant on the levels of micronutrients (Cu, Fe, Zn, Mn, and B). Contrarily, the interaction effect of the cultivation system and micronutrient spray on the concentration of any of the micronutrients was not significant. The highest levels of Zn, Fe, Mn, and B ( $53.36 \pm 3.94$ ,  $74.08 \pm 8.75$ ,  $94.3 \pm 8.63$ , and  $5.66 \pm 1.44 \mu\text{g/g}$ , respectively) were found in the hydroponic system. The lowest levels of all these elements were observed in field cultivation. However, the highest level of Cu ( $7.94 \pm 1.48 \mu\text{g/g}$ ) was observed in the soil culture of the greenhouse and the lowest in the hydroponic system and the field (Table 2).

The mean comparison of the influence of foliar spraying of micronutrients on the levels of micronutrients in the aerial parts of basil is shown in Table 3. The highest level of Cu ( $12.91 \pm 2.38 \mu\text{g/g}$ ) was observed in the treatment of Cu foliar spray, which was 83.64% higher compared to the control (water spray). The lowest level of Cu ( $6.35 \pm 1.46 \mu\text{g/g}$ ) was observed following Fe foliar spray. The highest level of Zn ( $54.02 \pm 4.75 \mu\text{g/g}$ ) was observed in the zinc foliar spray treatment, which was 41.71% higher compared to the control treatment (water spray). The lowest level of Zn was obtained in the Cu foliar spray treatment. Fe foliar application increased the level of Fe in the aerial parts of basil by 56.54% compared to the control. The highest level of Fe ( $94.95 \pm 8.37 \mu\text{g/g}$ ) was observed in the treatment under Fe application. The foliar spray of Mn significantly increased the content of Mn in the aerial parts of basil. The highest level of Mn ( $90.30 \pm 8.63 \mu\text{g/g}$ ) was obtained in the treatment with Mn foliar spray, which showed an increase of 74.86% compared to the treatment of water spray. The highest level of B ( $7.05 \pm 2.08 \mu\text{g/g}$ ) was observed in the treatment of B spray, which was 44.05% higher compared to the control treatment (Table 3).

**Table 3.** Mean comparison of the effect of foliar spraying of micronutrients on the concentration of some microelements in basil leaves.

| Foliar Spray        | Cu                 | Zn                    | Fe                 | Mn                 | B                 |
|---------------------|--------------------|-----------------------|--------------------|--------------------|-------------------|
| ( $\mu\text{g/g}$ ) |                    |                       |                    |                    |                   |
| Control             | $7.03 \pm 2.11^b$  | $38.12 \pm 3.55^{bc}$ | $61.43 \pm 6.44^b$ | $51.64 \pm 3.74^b$ | $4.88 \pm 1.38^b$ |
| Cu                  | $12.91 \pm 2.38^a$ | $33.38 \pm 4.21^c$    | $59.56 \pm 6.65^b$ | $49.05 \pm 6.43^b$ | $4.28 \pm 1.22^b$ |
| Zn                  | $7.23 \pm 1.34^b$  | $54.02 \pm 4.75^a$    | $60.34 \pm 7.74^b$ | $48.83 \pm 4.65^b$ | $4.30 \pm 1.11^b$ |
| Mn                  | $6.59 \pm 1.14^b$  | $42.18 \pm 5.78^b$    | $58.42 \pm 4.73^b$ | $90.30 \pm 8.62^a$ | $4.29 \pm 0.84^b$ |
| Fe                  | $6.35 \pm 1.46^b$  | $36.52 \pm 4.66^{bc}$ | $94.95 \pm 8.37^a$ | $46.98 \pm 7.55^b$ | $4.30 \pm 0.78^b$ |
| B                   | $7.30 \pm 1.55^b$  | $40.89 \pm 4.26^{bc}$ | $59.33 \pm 6.36^b$ | $50.67 \pm 7.71^b$ | $7.05 \pm 2.08^a$ |

Means with the same letters in each column do not have a significant difference at the 1% probability level based on the LSD test.

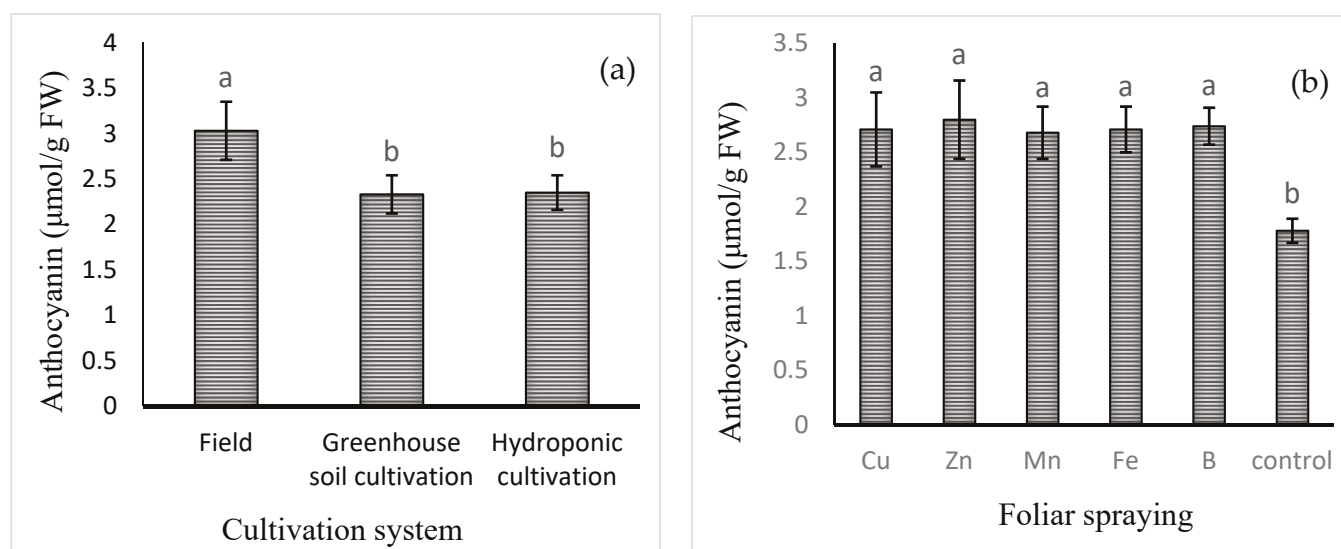
#### 2.4. Phytochemistry

The analysis of variance (Table S1) revealed that the simple effects of the cultivation system and foliar spraying of micronutrients on the levels of total anthocyanins, phenolics, and flavonoids, antioxidant properties by FRAP and DPPH methods, and essential oil content and yield were significant. The effect of cultivation system  $\times$  foliar spraying was also significant on total phenolics and flavonoids, antioxidant properties by FRAP and DPPH methods, and essential oil yield.

##### 2.4.1. Anthocyanins

The mean comparisons of the effect of the cultivation system on the levels of anthocyanins are shown in Figure 2a. The highest content of anthocyanin ( $3.03 \pm 0.32 \mu\text{mol/g FW}$ ) was found in field cultivation and the lowest in the hydroponic ( $2.33 \pm 0.21 \mu\text{mol/g FW}$ ) and soil greenhouse ( $2.35 \pm 0.19 \mu\text{mol/g FW}$ ) cultivation systems. Among the foliar applications of different micronutrients, the highest level of anthocyanin was found in the Zn treatment ( $2.80 \pm 0.36 \mu\text{mol/g FW}$ ), which was not significantly different from that of the B, Cu, Fe, or Mn treatment. The lowest level of anthocyanin was observed in the control treatment ( $1.78 \pm 0.11 \mu\text{mol/g FW}$ ).





**Figure 2.** Mean comparison of the effects of (a) cultivation system and (b) foliar spraying of micronutrients on anthocyanin content of basil. Different lowercase letters indicate significant differences among different treatments.

#### 2.4.2. Total Phenolic and Flavonoid Content

The mean comparisons of the effect of the cultivation system  $\times$  foliar spray on the content of total phenolics showed that the highest level ( $16.54 \pm 2.42$  mg GAE/g DW) was observed in field cultivation under the Zn foliar application. The lowest level of total phenolics was found under the hydroponic cultivation system with the control, Cu, and Mn treatments ( $1.57 \pm 0.42$ ,  $2.4 \pm 0.62$ , and  $2.64 \pm 0.18$  mg GAE/g DW, respectively). The highest level of flavonoids ( $9.65 \pm 2.01$  mg QU/g DW) was observed in field cultivation with Zn foliar treatment. The lowest level of flavonoids ( $0.64 \pm 0.12$  mg of QU/g DW) was observed in the hydroponic cultivation system with no foliar spray, which was not significantly different from the Cu or Mn foliar spray in the hydroponic cultivation system. Generally, the plants grown in the field possessed higher contents of total phenolics and flavonoids compared to greenhouse cultivation (Table 1).

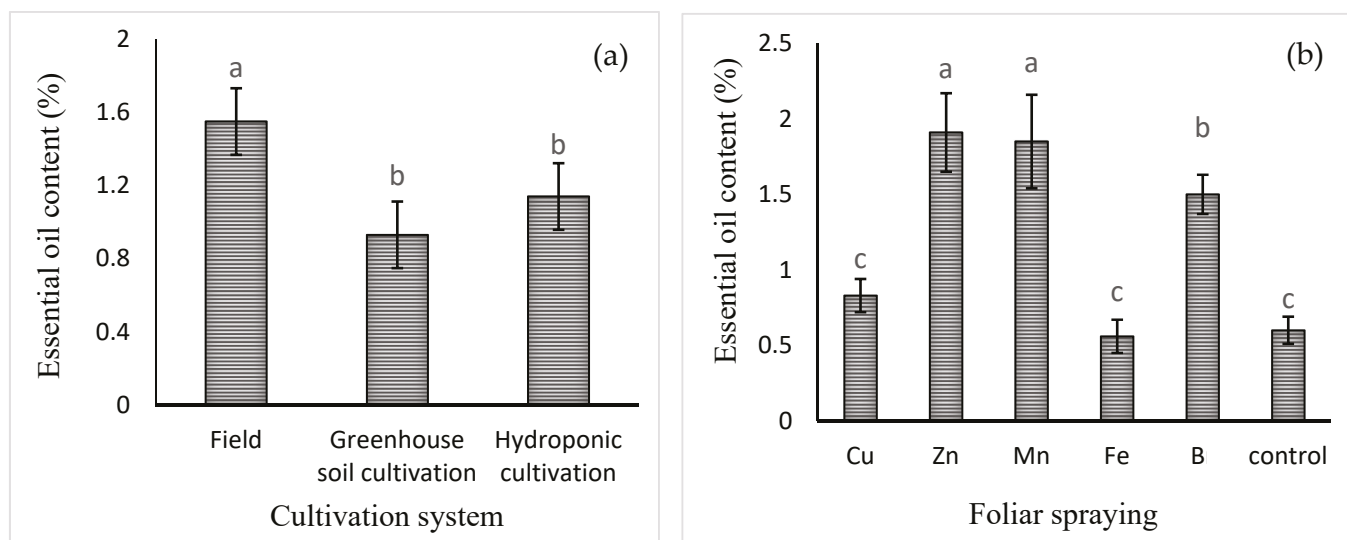
#### 2.4.3. Antioxidant Activity

The mean comparisons of the cultivation system  $\times$  foliar spraying of micronutrients showed that the highest antioxidant activity, measured with the DPPH method, was found in the field cultivation system with foliar spray of Zn, Mn, or B (with  $IC_{50}$  equal to  $5.41 \pm 1.1$ ,  $5.84 \pm 0.87$ , and  $5.99 \pm 1.42$  mg/mL). The lowest antioxidant activity was recorded for hydroponic culture with water spray or Cu foliar spray (with  $IC_{50}$  equal to  $10.48 \pm 2.01$  and  $10.25 \pm 1.7$  mg/mL, respectively).  $IC_{50}$  represents the concentration of the extract that inhibits free radicals by 50%, so a lower  $IC_{50}$  indicates a higher antioxidant property. The highest level of antioxidants, measured with the FRAP method, was observed in the field cultivation system under the foliar spray of Zn, Mn, or B ( $7.26 \pm 1.02$ ,  $7.15 \pm 1.14$ , or  $7.08 \pm 1.13$  mmol Fe/g DW, respectively). In addition, it was found that plants grown in the field showed higher antioxidant activity, regardless of the measurement method (Table 1).

#### 2.4.4. Essential Oil Content and Yield

The mean comparisons of the effect of the cultivation system on the content of basil essential oil (expressed in percentage) are shown in Figure 3a. The highest level of essential oil was obtained in field cultivation ( $1.55 \pm 0.23\%$  w/w). Foliar application of micronutrients, except for that of Fe or Cu, increased the essential oil percentage. Foliar application of Zn, Mn, or B increased the content of essential oil by 218, 208, and 150%, respectively,

compared to the control (Figure 3b). The mean comparisons of the effect of the cultivation system  $\times$  foliar spray on essential oil yield are shown in Table 2. The highest essential oil yield ( $0.3 \pm 0.05$  g/plant) was noted in field cultivation with Zn foliar spray, though this was not significantly different from the yield in hydroponic culture treated with Mn foliar spray. In the comparison between cultivation systems, it was found that there was no significant difference between field and hydroponic cultivation in terms of essential oil yield.



**Figure 3.** Mean comparison of the effects of (a) cultivation system and (b) foliar spraying of micronutrients on basil essential oil content (%). Different lowercase letters indicate significant differences among different treatments.

#### 2.4.5. Essential Oil Compounds

GC-MS analysis of basil essential oil identified 31 compounds (Table 4). Methyl chavicol (average: 43.08%), linalool (average: 25.46%), and *epi*- $\alpha$ -cadinol (average: 9.2%) were identified as the main components of basil essential oil. In addition, germacrene-D,  $\alpha$ -bergamotene, 1,8-cineole, and  $\gamma$ -cadinene were other important components of the essential oil. The effect of the cultivation system on essential oil compounds, including 1,8-cineole, linalool, methyl chavicol,  $\alpha$ -bergamotene,  $\gamma$ -cadinene,  $\delta$ -cadinene, spathulenol, 1,10-di-*epi*-cubenol, and *epi*- $\alpha$ -cadinol, was significant. The percentages of 1,8-cineole,  $\alpha$ -bergamotene,  $\gamma$ -cadinene, caryophyllene oxide, 1,10-di-*epi*-cubenol, and *epi*- $\alpha$ -cadinol changed significantly following the foliar application of micronutrients. The effect of the cultivation system  $\times$  micronutrient foliar application was also significant in the case of 1,8-cineole, methyl chavicol,  $\alpha$ -bergamotene,  $\gamma$ -cadinene, caryophyllene oxide, 1,10-di-*epi*-cubenol, and *epi*- $\alpha$ -cadinol (Table S1).

Mean comparisons of the effect of the cultivation system are shown in Figure 4. The highest levels of linalool,  $\delta$ -cadinene, and spathulenol ( $32.27 \pm 2.8\%$ ,  $1.18 \pm 0.09\%$ , and  $1.48 \pm 0.23\%$ , respectively) were obtained in the greenhouse soil culture (Figure 4). Mean comparisons of the effect of the cultivation system  $\times$  foliar spraying are shown in Table 5. The highest percentage of 1,8-cineole was noted in the field cultivation under Cu and Zn treatments ( $6.29 \pm 1.08$  and  $6.8 \pm 1.11\%$ , respectively). However, the level of 1,8-cineole in hydroponic culture was very low, regardless of the foliar spraying treatment. The highest level of methyl chavicol, the main component of basil essential oil, was observed in all treatments with foliar application of micronutrients in hydroponic cultivation. However, there was no significant difference with Mn or B foliar spraying in the hydroponic cultivation. The level of methyl chavicol in hydroponic culture was significantly higher than in soil culture in the greenhouse and in the field. The highest percentage of caryophyllene oxide ( $2.26 \pm 0.14\%$ ) was observed under Fe foliar application in field cultivation. Furthermore, the highest percentage of  $\alpha$ -bergamotene ( $3.76 \pm 1.01\%$ ) was observed in

hydroponic culture with water spraying. The highest level of  $\delta$ -cadinene was observed in the control ( $2.65 \pm 0.38\%$ ) and/or B spray ( $2.2 \pm 0.23\%$ ) treatments in the greenhouse soil culture and in the Fe foliar treatment in the field culture ( $2.03 \pm 0.28\%$ ). However, the highest percentage of 1,10-di-*epi*-cubenol ( $5.1 \pm 1.01\%$ ) was obtained in the field cultivation system with Fe application. The highest percentage of *epi*- $\alpha$ -cadinol ( $24.01 \pm 3.78\%$ ) was obtained in greenhouse soil culture with Zn foliar application. Finally, the percentage of *epi*- $\alpha$ -cadinol in greenhouse soil culture was much higher than in field soil culture and hydroponic culture (Table 5).

**Table 4.** Constituents of the essential oil in basil.

| No | Oil Constituents               | RI <sup>a</sup> | LIT RI <sup>b</sup> | ID <sup>c</sup> | No | Oil Constituents               | RI   | KI      | ID <sup>c</sup> |
|----|--------------------------------|-----------------|---------------------|-----------------|----|--------------------------------|------|---------|-----------------|
| 1  | $\beta$ -Myrcene               | 988             | 987.03              | RI, MS          | 16 | $\gamma$ -Muurolene            | 1449 | 1454.83 | RI, MS          |
| 2  | 1,8-Cineole                    | 1031            | 1030.11             | Std             | 17 | Z- $\beta$ -Farnesene          | 1460 | 1458.14 | RI, MS          |
| 3  | ( <i>E</i> )- $\beta$ -Ocimene | 1040            | 1041.43             | RI, MS          | 18 | Germacrene D                   | 1474 | 1473.74 | Std             |
| 4  | Fenchone                       | 1088            | 1094.28             | RI, MS          | 19 | $\alpha$ -Selinene             | 1498 | 1500    | RI, MS          |
| 5  | Linalool                       | 1101            | 1102.99             | Std             | 20 | $\alpha$ -Bulnesene            | 1509 | 1495.36 | RI, MS          |
| 6  | Camphor                        | 1146            | 1150.07             | RI, MS          | 21 | $\gamma$ -Cadinene             | 1514 | 1506.84 | RI, MS          |
| 7  | Borneol                        | 1164            | 1166.13             | RI, MS          | 22 | $\delta$ -Cadinene             | 1525 | 1512.63 | RI, MS          |
| 8  | Terpinen-4-ol                  | 1177            | 1184.16             | RI, MS          | 23 | ( <i>E</i> )-Nerolidol         | 1554 | 1557.69 | RI, MS          |
| 9  | Methyl chavicol                | 1200            | 1207.62             | Std             | 24 | Spathulenol                    | 1572 | 1568.52 | RI, MS          |
| 10 | $\beta$ -Copaene               | 1378            | 1370.31             | RI, MS          | 25 | Caryophyllene oxide            | 1573 | 1571.84 | Std             |
| 11 | Bornyl acetate                 | 1283            | 1283.18             | RI, MS          | 26 | Isoaromadendrene epoxide       | 1579 | 1574.05 | RI, MS          |
| 12 | Methyl eugenol                 | 1400            | 1402.32             | Std             | 27 | 1,10-di- <i>epi</i> -Cubenol   | 1601 | 1606.67 | RI, MS          |
| 13 | ( <i>E</i> )-Caryophyllene     | 1415            | 1413.84             | RI, MS          | 28 | <i>epi</i> - $\alpha$ -Cadinol | 1644 | 1635.45 | RI, MS          |
| 14 | $\alpha$ -Bergamotene          | 1430            | 1427.91             | RI, MS          | 29 | $\beta$ -Eudesmol              | 1649 | 1644.82 | RI, MS          |
| 15 | $\beta$ -Gurjunene             | 1449            | 1443.71             | RI, MS          | 30 | $\alpha$ -Bisabolol            | 1691 | 1681.46 | RI, MS          |

<sup>a</sup> Linear retention indices on the HP-5 column, which were experimentally determined using a homologous series of n-alkanes. <sup>b</sup> Relative retention indices, which were taken from Adams. <sup>c</sup> Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries (Wiley, Adams, and NIST 05); RI: by comparison of the retention index with those reported in the literature; Std: by comparison of the retention time and mass spectrum of an available authentic standard.

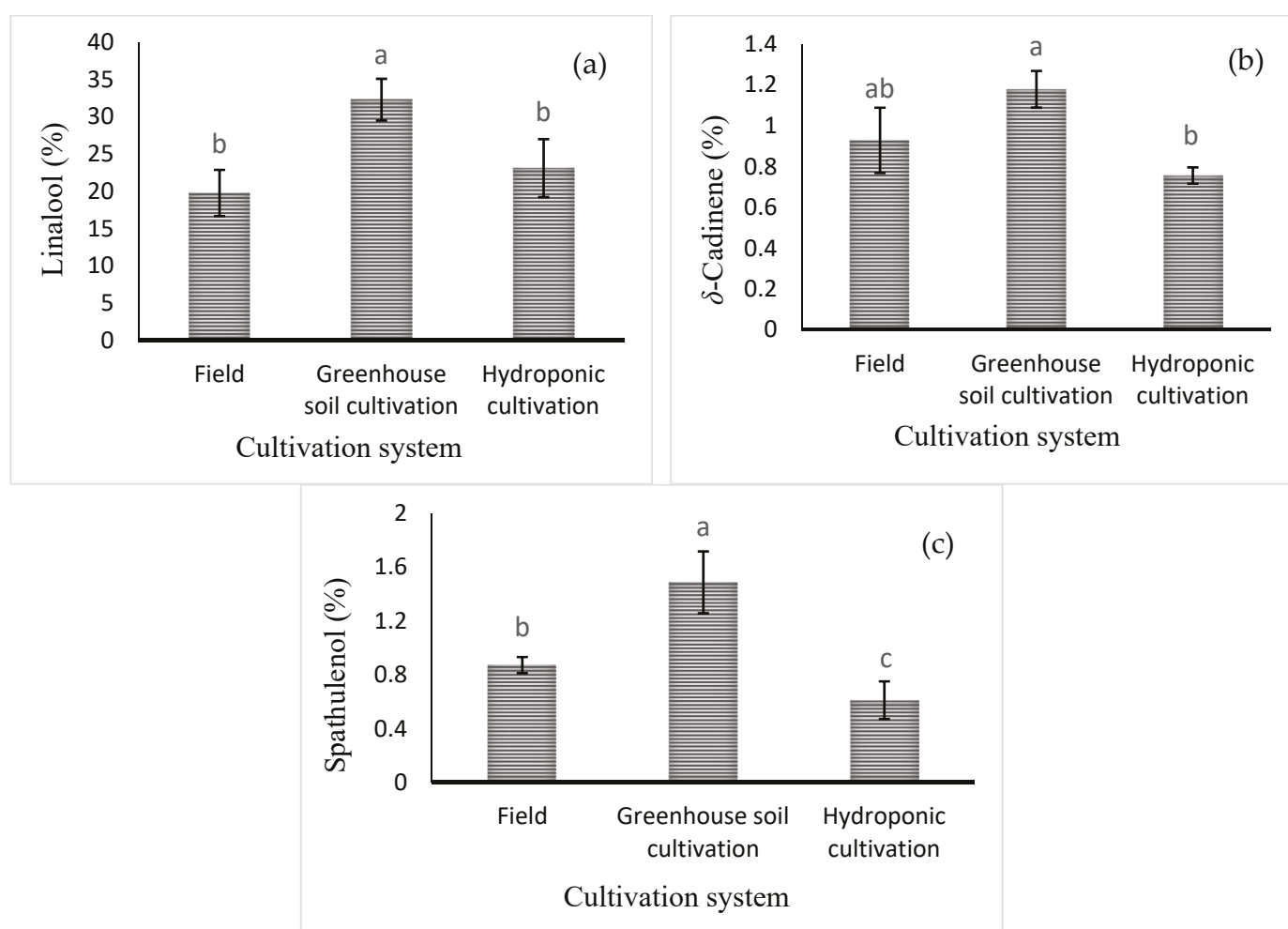
**Table 5.** Mean comparison of the interaction effect of cultivation system and foliar spraying on basil essential oil constituents.

| Cultivation System      | Foliar Spray | 1,8-Cineole                   | Methyl Chavicol               | $\alpha$ -Bergamotene         | $\gamma$ -Cadinene           | Caryophyllene Oxide           | 1,10-di- <i>epi</i> -Cubenol  | <i>epi</i> - $\alpha$ -Cadinol |
|-------------------------|--------------|-------------------------------|-------------------------------|-------------------------------|------------------------------|-------------------------------|-------------------------------|--------------------------------|
| Field                   | Control      | $1.41 \pm 0.38$<br>de         | $43.08 \pm 2.72$<br>bcd       | $2.62 \pm 0.58$<br>a-d        | $0.95 \pm 0.08$ <sup>b</sup> | $1.34 \pm 0.03$ <sup>b</sup>  | $1.14 \pm 0.06$ <sup>bc</sup> | $8.89 \pm 1.03$<br>def         |
|                         | Cu           | $6.29 \pm 1.08$ <sup>a</sup>  | $40.09 \pm 4.62$<br>cde       | $2.61 \pm 0.67$<br>a-d        | $0.8 \pm 0.06$ <sup>b</sup>  | $0.69 \pm 0.02$ <sup>bc</sup> | $0.96 \pm 0.04$ <sup>bc</sup> | $6.57 \pm 1.02$<br>fgh         |
|                         | Zn           | $6.80 \pm 1.11$ <sup>a</sup>  | $41.49 \pm 3.77$<br>b-e       | $2.69 \pm 0.85$<br>a-d        | $0.93 \pm 0.05$ <sup>b</sup> | $1.16 \pm 0.28$ <sup>bc</sup> | $1.27 \pm 0.13$ <sup>bc</sup> | $7.86 \pm 1.11$<br>efg         |
|                         | Mn           | $4.50 \pm 0.58$ <sup>b</sup>  | $51.62 \pm 5.62$<br>abc       | $2.63 \pm 0.93$<br>a-d        | $0.52 \pm 0.03$ <sup>b</sup> | $0.77 \pm 0.13$ <sup>bc</sup> | $0.72 \pm 0.15$ <sup>c</sup>  | $5.03 \pm 0.58$ <sup>hi</sup>  |
|                         | Fe           | $2.79 \pm 0.28$<br>cd         | $24.21 \pm 3.55$ <sup>f</sup> | $3.04 \pm 1.01$<br>ab         | $2.03 \pm 0.28$ <sup>a</sup> | $2.26 \pm 0.14$ <sup>a</sup>  | $5.10 \pm 1.01$ <sup>a</sup>  | $10.37 \pm 1.44$<br>cde        |
|                         | B            | $3.66 \pm 0.73$ <sup>bc</sup> | $46.32 \pm 5.72$<br>abc       | $1.62 \pm 0.28$<br>c-f        | $0.69 \pm 0.04$ <sup>b</sup> | $1.06 \pm 0.08$ <sup>bc</sup> | $1.11 \pm 0.04$ <sup>bc</sup> | $5.89 \pm 1.02$<br>ghi         |
| Greenhouse soil culture | Control      | $0.87 \pm 0.08$ <sup>e</sup>  | $30.21 \pm 4.11$<br>ef        | $1.92 \pm 0.25$<br>b-f        | $2.65 \pm 0.38$ <sup>a</sup> | $1.31 \pm 0.09$ <sup>b</sup>  | $2.57 \pm 0.12$ <sup>b</sup>  | $9.42 \pm 1.04$<br>de          |
|                         | Cu           | $0.66 \pm 0.12$ <sup>e</sup>  | $31.09 \pm 4.45$<br>def       | $2.67 \pm 0.28$<br>a-d        | $0.41 \pm 0.08$ <sup>b</sup> | $1.12 \pm 0.05$ <sup>bc</sup> | $1.32 \pm 0.04$ <sup>bc</sup> | $18.88 \pm 2.52$<br>b          |
|                         | Zn           | $0.60 \pm 0.18$ <sup>e</sup>  | $27.15 \pm 3.32$ <sup>f</sup> | $1.36 \pm 0.22$ <sup>ef</sup> | $0.34 \pm 0.03$ <sup>b</sup> | $0.55 \pm 0.03$ <sup>c</sup>  | $1.73 \pm 0.16$ <sup>bc</sup> | $24.01 \pm 3.78$<br>a          |
|                         | Mn           | $0.81 \pm 0.09$ <sup>e</sup>  | $32.34 \pm 3.31$<br>def       | $1.59 \pm 0.29$<br>def        | $0.78 \pm 0.09$ <sup>b</sup> | $1.32 \pm 0.18$ <sup>b</sup>  | $1.74 \pm 0.48$ <sup>bc</sup> | $12.17 \pm 1.88$<br>c          |
|                         | Fe           | $0.61 \pm 0.05$ <sup>e</sup>  | $31.83 \pm 2.61$<br>def       | $1.29 \pm 0.34$ <sup>ef</sup> | $0.67 \pm 0.12$ <sup>b</sup> | $1.06 \pm 0.11$ <sup>bc</sup> | $1.8 \pm 0.23$ <sup>bc</sup>  | $11.15 \pm 1.95$<br>cd         |
|                         | B            | $0.61 \pm 0.11$ <sup>e</sup>  | $33.2 \pm 4.63$<br>def        | $1.40 \pm 0.38$ <sup>ef</sup> | $2.2 \pm 0.23$ <sup>a</sup>  | $0.69 \pm 0.05$ <sup>bc</sup> | $1.13 \pm 0.14$ <sup>bc</sup> | $17.08 \pm 2.58$<br>b          |

Table 5. Cont.

| Cultivation System | Foliar Spray | 1,8-Cineole                  | Methyl Chavicol                 | $\alpha$ -Bergamotene          | $\gamma$ -Cadinene           | Caryophyllene Oxide           | 1,10-di- <i>epi</i> -Cubenol  | <i>epi</i> - $\alpha$ -Cadinol |
|--------------------|--------------|------------------------------|---------------------------------|--------------------------------|------------------------------|-------------------------------|-------------------------------|--------------------------------|
| Hydroponic culture | Control      | 0.62 $\pm$ 0.06 <sup>e</sup> | 51.06 $\pm$ 5.64 <sup>abc</sup> | 3.76 $\pm$ 1.01 <sup>a</sup>   | 0.86 $\pm$ 0.05 <sup>b</sup> | 1.05 $\pm$ 0.07 <sup>bc</sup> | 0.93 $\pm$ 0.11 <sup>bc</sup> | 5.58 $\pm$ 0.56 <sup>ghi</sup> |
|                    | Cu           | 0.6 $\pm$ 0.12 <sup>e</sup>  | 55.86 $\pm$ 5.75 <sup>ab</sup>  | 2.45 $\pm$ 0.08 <sup>b-e</sup> | 1.03 $\pm$ 0.08 <sup>b</sup> | 0.84 $\pm$ 0.03 <sup>bc</sup> | 0.58 $\pm$ 0.08 <sup>c</sup>  | 3.44 $\pm$ 0.78 <sup>i</sup>   |
|                    | Zn           | 0.66 $\pm$ 0.07 <sup>e</sup> | 57.36 $\pm$ 5.96 <sup>a</sup>   | 2.82 $\pm$ 0.09 <sup>abc</sup> | 0.6 $\pm$ 0.04 <sup>b</sup>  | 0.98 $\pm$ 0.08 <sup>bc</sup> | 0.72 $\pm$ 0.09 <sup>c</sup>  | 4.75 $\pm$ 1.04 <sup>hi</sup>  |
|                    | Mn           | 0.6 $\pm$ 0.11 <sup>e</sup>  | 52.14 $\pm$ 6.75 <sup>abc</sup> | 2 $\pm$ 0.25 <sup>b-f</sup>    | 0.87 $\pm$ 0.06 <sup>b</sup> | 0.96 $\pm$ 0.06 <sup>bc</sup> | 0.77 $\pm$ 0.12 <sup>bc</sup> | 5.51 $\pm$ 0.48 <sup>ghi</sup> |
|                    | Fe           | 0.67 $\pm$ 0.13 <sup>e</sup> | 58.54 $\pm$ 6.84 <sup>a</sup>   | 1.9 $\pm$ 0.18 <sup>b-f</sup>  | 0.4 $\pm$ 0.02 <sup>b</sup>  | 1.08 $\pm$ 0.13 <sup>bc</sup> | 0.43 $\pm$ 0.04 <sup>c</sup>  | 3.78 $\pm$ 0.45 <sup>i</sup>   |
|                    | B            | 0.67 $\pm$ 0.10 <sup>e</sup> | 53.64 $\pm$ 4.81 <sup>ab</sup>  | 2.34 $\pm$ 0.32 <sup>b-e</sup> | 0.77 $\pm$ 0.18 <sup>b</sup> | 0.69 $\pm$ 0.04 <sup>bc</sup> | 0.88 $\pm$ 0.09 <sup>bc</sup> | 3.67 $\pm$ 0.66 <sup>ghi</sup> |

Means with the same letters in each column do not have a significant difference at the 1% probability level based on the LSD test.



**Figure 4.** Mean comparison of the effect of cultivation system on (a) linalool, (b)  $\delta$ -cadinene, and (c) spathulenol relative percentages in basil essential oil. Different lowercase letters indicate significant differences among different treatments.

### 3. Discussion

In the present study, basil plants cultivated in a hydroponic system had higher plant height, leaf dry weight, and leaf area. Contrarily, basil plants grown in the field were woodier than those grown in the soil in the greenhouse and in the hydroponic system, resulting in a higher stem dry weight. The better growth and higher yield of basil in the hydroponic system may be attributed to better aeration of the root zone, which leads to

better plant root activity. In addition, the presence of sufficient nutrients in the growing media and increased absorption by the roots leads to the improvement of the photosynthetic efficiency, and, therefore, increases the growth and biomass of the plant. The higher relative humidity of the greenhouse and optimal absorption of water by the roots in the hydroponic system also improve the water balance of the plant and, therefore, increase the division and enlargement of the leaf mesophyll cells (higher leaf area) and increase the content of chlorophyll [25]. The results of the present study also showed that the uptake of nutrients such as K, Ca, Mg, N, P, Mn, Fe, B, and Zn in the hydroponic cultivation system was much better than in the greenhouse soil cultivation and field cultivation systems. Therefore, the better nutritional status of the plant in hydroponic conditions leads to higher growth and yield. The increase in the absorption of mineral elements in soilless cultivation can be due to the optimal concentration of these elements, increasing root access to nutrients due to the suitable pH of the nutrient solution and culture medium. In the case of less mobile elements such as Ca, the absorption is strongly influenced by the humidity of the substrate and the access of the plant roots to water. The optimal availability and solubility of Ca in the culture medium may result from the improvement of the water content of the medium, thereby increasing Ca absorption in the soilless system [26,27]. In a study comparing the vegetative and physiological characteristics of *Aloysia citriodora* Palau in two cultivation systems (soil and hydroponics), it was found that the plants grown in the hydroponic system had a higher stem dry weight, root dry weight, plant dry weight, shoot length, and shoot fresh weight compared to soil grown plants [28]. In another trial, lavender, garden thyme, and marigold plants were compared in hydroponic/air and soil cultivation systems in the greenhouse. The results showed that plant growth and yield indices were higher in hydroponic and air cultivation systems than in a soil cultivation system [29].

Different cultivation systems affect the concentration of bioactive compounds in medicinal plants differently [30]. In the present study, the content of total phenolics, flavonoids, and anthocyanins, the antioxidant capacity of the extract, and the percentage of basil essential oil in the field were higher than in greenhouse cultivation systems (soil and hydroponics). However, there was no significant difference in the yield of essential oil between field cultivation and hydroponic systems. Considering that essential oil yield depends on both the percentage of essential oil and plant biomass [7], the high yield of essential oil in the field and the high yield of plant biomass in the hydroponic system led to similar essential oil yield. Therefore, there was no significant difference between the two systems. Contrarily, Giurgiu et al. [29] reported that lavender and garden thyme plants grown in hydroponic systems had 20–30% more essential oil yield than those grown in the field. These differences in the responses of plants could be due to factors such as the inherent characteristics of the species, plant origin, experimental conditions, and different types of hydroponic systems [5,30].

Phenolic compounds act effectively as hydrogen donors and are considered important antioxidants [31]. Given the higher levels of phenolics, flavonoids, and anthocyanins in basil cultivated in the field, the high antioxidant activity in field-grown plants is unsurprising [32]. Studies have shown that lack of water combined with high light intensity (field conditions) leads to the closure of the stomata, as a result of which the absorption of carbon dioxide and its stabilization is reduced. Following this, the consumption of  $\text{NADPH} + \text{H}^+$  to stabilize carbon dioxide decreases during the Calvin cycle, which leads to the accumulation of high levels of  $\text{NADPH} + \text{H}^+$  in the cell. In such conditions, the production of highly reduced natural compounds (secondary metabolites) that require high consumption of  $\text{NADPH} + \text{H}^+$  increases [33]. In fact, the biosynthesis of secondary metabolites may be an auxiliary system to release excess energy in conditions of stomatal closure in plants under stress. Therefore, regardless of their ecological effects, secondary metabolites can play a vital role in the plant life cycle as a mechanism for releasing excess energy [34]. Additionally, most secondary metabolites play an essential role in increasing the resistance of plants to biotic and abiotic stresses. Therefore, the increase in the levels of phenolics, flavonoids, anthocyanins, and essential oil in field cultivation can be due to



some limitations, such as unsuitable soil properties, unfavorable weather conditions, and lack of proper root access to water and nutrients compared to greenhouse cultivation [35]. In *Artemisia dracuncululus* L., despite the decrease in vegetative growth, water deficit stress increased both the percentage and yield of essential oil, the contents of total phenolics, total flavonoids, and antioxidant properties compared to the control plants [5]. Jaafar et al. [36] reported that water deficit stress not only increased the total phenolic and flavonoid content of *Marantodes pumilum* (Blume) Kuntze but also increased their yield. In the comparison of parsley plants in different cultivation systems (field, greenhouse, and tunnel cultivation), the highest level of ascorbic acid was obtained in the field. The level of ascorbic acid in the field was 2.6 and 5.4 times higher than in tunnel cultivation and greenhouse cultivation, respectively [30]. A comparison of ginseng (*Panax ginseng* C.A.Mey.) seedlings cultivated in hydroponic and soil cultivation systems showed that total phenolic and flavonoid levels in soil culture were higher than in hydroponic culture [37]. The level of carlina oxide in *C. acaulis* plants grown in the field was significantly higher than in those grown under hydroponic conditions. The flavonoid content of plant leaves was similar between the two cultivation systems. However, the antioxidant capacity and concentration of secondary metabolites were higher in the soil cultivation system. The large difference in carlina oxide content between soil-grown and hydroponically grown plants was attributed to differences in plant growth conditions, such as water supply, nutritional status, temperature, relative humidity, and light intensity [15]. Braglia et al. [38] reported that total phenolic content and antioxidant activity were significantly higher in aquaponically grown basil plants compared to soil-grown organic basil plants.

In the present study, the highest levels of linalool,  $\delta$ -cadinene, and spathulenol were observed in greenhouse soil cultivation. The level of 1,8-cineole was very low in the hydroponic culture. However, the level of methyl chavicol, the principal essential oil constituent, in hydroponic cultivation was higher than in field and greenhouse soil cultivations. Generally, most medicinal and industrial applications of basil depend on its essential oil, especially the methyl chavicol content. Methyl chavicol is commonly used as a flavoring agent in food and beverages [39]. Alvarenga et al. [40] showed that nutrition affects the essential oil content in yarrow plants in the hydroponic system. Low levels of Ca reduced the growth of *Achillea millefolium* L. more than those of other studied elements, while the removal of S, P, Mn, or B promoted the essential oil percentage. Moreover, their results showed that the removal of macronutrients or micronutrients may be beneficial, as it can boost the percentage of some volatile compounds. For example, the sabinene percentage increased with Mg removal and the cubebene percentage with P removal. The same can be observed by removing Zn, Fe, or Mo to promote the level of (*E*)-caryophyllene, a compound with anti-inflammatory activity. In the current study, the changes in the levels of basil essential compounds in different cultivation systems can be due to the change in the levels of nutrients available to the plant in different cultivation conditions.

Foliar application of micronutrients in each of the cultivation systems yielded different results. In the field, the highest plant height, stem dry weight, and leaf area were noted in Fe foliar application; in greenhouse soil cultivation, the highest plant height was observed with the application of B or Fe, the highest dry weight of the stem was observed with the application of Zn, and the largest leaf area was observed with the application of Mn; in hydroponic culture, the highest plant height was noted with the use of B. Overall, in field cultivation and hydroponic cultivation, the application of any micronutrient increased stem dry weight and leaf area compared to the control treatment. However, no significant change was observed among the elements. It seems that the difference in the level of nutrients in the field soil, greenhouse soil, and nutrient solution in the hydroponic system explains the different responses of basil to the foliar application of elements in different cultivation substrates. Micronutrients play a prominent role in the growth and metabolism of plants. Therefore, their deficiency may cause several physiological disorders in plants and thus reduce their yield and quality [41]. Considering the role of B in increasing the longitudinal growth and cell division in plants, plant growth and biomass production are expectedly

enhanced following the application of B. In addition, Zn and B are particularly important in plant growth and development because they are essential in the synthesis of plant growth regulators [6]. The increase in plant growth parameters following the application of Fe, Mn, or Cu is also due to their vital role in many enzymes involved in physiological and biochemical processes [42].

In the present study, under field conditions, the highest levels of total phenolics, total flavonoids, and antioxidant capacity were observed following Zn or Mn foliar application. In hydroponic cultivation, foliar spraying of Zn or B led to the highest antioxidant capacity and levels of phenolics and flavonoids. Regardless of the cultivation system, foliar spraying of Cu, Mn, Zn, Fe, or B significantly increased basil anthocyanin content. Micronutrients such as Zn and Fe play an effective role in preserving antioxidants and increasing anthocyanin content. Additionally, Mn plays a very important role in the production of anthocyanins [43]. It has been reported that Fe, Mn, and Zn foliar application increased the total phenolic content of *Borago officinalis* L. [44]. Nutrient accessibility in the culture medium is an important factor in changing the yield and quality of the essential oil. Planting methods, climatic conditions, fertilization, irrigation, and harvesting date of plant material can change the yield and composition of the essential oil [45]. In the present study, regardless of the cultivation system, the highest essential oil percentage was noted in the treatment of foliar spraying with Zn or Mn. In field conditions and greenhouse soil cultivation, Zn, followed by Mn, foliar application led to the highest yield of essential oil. In hydroponic cultivation, the highest essential oil yield was observed following Mn, followed by Zn, foliar application. The highest level of methyl chavicol was found in hydroponic cultivation in all micronutrient treatments. In field conditions, Mn and B foliar spray resulted in the production of higher levels of methyl chavicol than other treatments. Considering the effects of B and Mn on plant growth and development, a reason for the increased levels of methyl chavicol in the essential oil seems to be the increased photosynthetic activity and the role of these elements in the chloroplast structure, which can lead to more production of essential oil secreting glands in the leaves [39]. Foliar spraying of Cu or Zn in the field also led to a significant increase in the level of cineol. In the greenhouse soil culture, the highest level of *epi*- $\alpha$ -cadinol was obtained in the essential oil of plants treated with Zn.

Kumara et al. [46] reported that treatment with Zn or Fe improved the quality of essential oil and increased the concentration of the main compounds of *Matricaria chamomilla* L. essential oil, such as chamazulene, Z-spiroether, and  $\alpha$ -bisabolol oxide A and B. In agreement with the results of the present study, foliar spraying of Zn or B significantly enhanced the biomass, percentage and yield of essential oil, total phenolics, flavonoids, and antioxidant capacity of *S. khuzistanica*. However, the spraying of Zn had no effect on carvacrol, the dominant component of *S. khuzistanica* essential oil [47]. The root yield of ginseng, total ginsenoside content, and nine types of ginsenosides in roots were significantly increased under foliar application of Fe, Zn, Mn, or Cu. In this study, the concentration of 500 mg/L of Fe, Mn, Cu, or Zn led to the highest yield and quality of ginseng [48]. Zn contributes to the synthesis of tryptophan, a precursor of indole acetic acid, and access to appropriate levels of this micronutrient can increase crop yield and quality [6]. It also acts as a metal component of various enzymes or as a structural, functional, and regulatory cofactor in relation to carbohydrate metabolism, photosynthesis, and protein production [49]. Since glucose and CO<sub>2</sub> are possible sources of carbon used in the biosynthesis of terpenes, the role of Zn in the production of essential oil seems to be very important. The spraying of micronutrients in mint plants caused a significant increase in plant growth and essential oil percentage and yield, as well as the level of menthol in the essential oil [50]. The foliar application of 50 mg/L of Mn or Zn and their combined application increased vegetative parameters, the essential oil percentage of seed, and the essential oil yield of the vegetative body and seed in cumin. The level of cumin-aldehyde, the dominant component of essential oil, also increased in seed essential oil and the vegetative body's essential oil [51].

Foliar application of micronutrients did not have a significant effect on the level of macronutrients (N, Ca, P, Mg, and K). However, foliar spraying of each of the micronutrients increased their concentration in the aerial parts of basil. Nutrients applied via foliar spraying penetrate leaf tissue through the cuticle or stomata, leading to an increased concentration of certain micronutrients in the leaves. Foliar spraying of micronutrients is an effective method for addressing specific deficiencies that may not be fully resolved through soil or hydroponic solution applications alone [22,43]. Fe foliar application not only increased the concentration of Fe in aerial parts but also decreased the concentration of Cu compared to the control. Therefore, the increase in Fe concentration in plants had a negative effect on Cu absorption. Foliar spraying of different concentrations of chitosan in red amaranth had no effect on the absorption of nutrients Ca, Mg, Na, K, and P but caused a significant increase in the absorption of S, Zn, and Fe [52]. Furthermore, manganese sulfate spraying on pomegranate shrubs increased the concentration of Mn and N in the leaves, while foliar spraying of zinc sulfate increased the concentration of Zn and decreased those of Mn and P in the leaves [53]. In chickpea plants, soil application or foliar application of Fe decreased the absorption and concentration of Mn in the aerial parts of the plant [42].

#### 4. Materials and Methods

##### 4.1. Planting Plants, Growing Conditions, and Applying Treatments

The present research was carried out in the farm and in the research greenhouse of the College of Agriculture and Natural Resources of Lorestan University over two consecutive years, 2020 and 2021 (longitude 48°26' E, latitude 33°44' N, altitude 1170 m). In this study, basil plants were compared in three cultivation systems, including cultivation in the field, cultivation in soil pots, and drip hydroponic cultivation. In each of the cultivation systems, foliar spraying of micronutrients was applied in a randomized complete block design. There were six treatments (control [water spray], Fe, Mn, Cu, Zn, and B foliar application), each with three replications. Perlite was used as the substrate in hydroponic cultivation (pH = 7.2, EC = 1.5). The sources of microelements were iron sulfate, manganese sulfate, copper sulfate, zinc sulfate, and boric acid. Before planting the plants, soil samples from the pots and the field were collected and analyzed for physical and chemical characteristics, as shown in Table 6 [54].

**Table 6.** Some physicochemical properties of field soil and pot substrate.

| Parameters (Units)                | Field Soil      | Pot Substrate   |
|-----------------------------------|-----------------|-----------------|
| pH                                | 7.13            | 6.9             |
| EC (dS m <sup>-1</sup> )          | 2.51            | 2.02            |
| Organic matter content (%)        | 1.74            | 2.8             |
| Organic carbon content (%)        | 1.04            | 1.4             |
| Total nitrogen (%)                | 0.15            | 0.24            |
| Potassium (mg kg <sup>-1</sup> )  | 331             | 362             |
| Phosphorus (mg kg <sup>-1</sup> ) | 12.4            | 14.76           |
| Sodium (mg kg <sup>-1</sup> )     | 78.2            | 66.8            |
| Magnesium (mg kg <sup>-1</sup> )  | 538.8           | 603.6           |
| Ferrite (mg kg <sup>-1</sup> )    | 5.18            | 4.44            |
| Textural class                    | Sandy clay loam | Sandy clay loam |

Basil seeds (purchased from Pakan Seed Co., Isfahan, Iran) were cultivated in a tray at a temperature of 18–22 °C and a relative humidity of 60% with 12 h of light and 12 h of darkness. After 3 weeks (May 5), the four-leaf seedlings of basil plants were transferred to the 5 L pots of the hydroponic system (perlite), the 5 L pots for the soil bed greenhouse cultivation, and the field cultivation. The plants in the hydroponic system were

fed with half-strength Hoagland's solution with a pH = 6, adjusted with HCL or NaOH ( $0.5 \text{ mol L}^{-1}$ ) (Table 7). For soil and hydroponic greenhouse cultivation, six pots were grown in each experimental unit. During the experiment, the average daily temperature of the greenhouse was 22–28 °C, the relative humidity was 60–70%, and the light intensity was  $600 \mu\text{mol/m}^2$  per second. For the field cultivation, six plots of  $2 \times 2 \text{ m}^2$  were prepared in each experimental block, each with 4 rows 50 cm apart. The plants were cultivated in the rows 20 cm apart. The distance between the plots was 1 m, and the distance between the blocks was 2 m. After planting, irrigation was carried out twice a week in the open field and soil culture in the greenhouse. Spraying of microelements at a concentration of 0.1% was conducted in all cultivation systems identically at 3 stages (6–8 real leaves, late vegetative growth, and the beginning of flowering) in the evening.

**Table 7.** Hoagland nutrient solution.

| Compounds     | MgSO <sub>4</sub> ·7H <sub>2</sub> O | Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O | KH <sub>2</sub> PO <sub>4</sub> | KNO <sub>3</sub> | H <sub>3</sub> BO <sub>3</sub> | MnCl <sub>2</sub> ·4H <sub>2</sub> O | ZnSO <sub>4</sub> ·7H <sub>2</sub> O | CuSO <sub>4</sub> ·5H <sub>2</sub> O | NaMoO <sub>3</sub> | Fe-DTPA            |
|---------------|--------------------------------------|------------------------------------------------------|---------------------------------|------------------|--------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------|--------------------|
| Unit          | mmol L <sup>−1</sup>                 |                                                      |                                 |                  |                                |                                      |                                      |                                      |                    | mg L <sup>−1</sup> |
| Concentration | 2                                    | 5                                                    | 1                               | 5                | 1                              | 1                                    | 1                                    | 1                                    | 1                  | 50                 |

#### 4.2. Reagents, Nutrients and Standards

Folin-Ciocalteu reagent, saturated sodium carbonate, aluminum chloride, potassium acetate, acetate buffer, ferric chloride, and iron sulfate were supplied by Sigma-Aldrich (St. Louis, MO, USA). Analytical grade 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrochloric acid, gallic acid, and rutin were purchased from Merck (Darmstadt, Germany).

Iron sulfate 99%, manganese sulfate 99.9%, copper sulfate 99.9%, zinc sulfate 99.9%, boric acid 99.9%, and Hoagland nutrient solution were obtained from Pars Oxide CO., Shiraz, Iran.

#### 4.3. Measurement of Plant Biomass

At the end of the experiment and in each cultivation system, five plants in each replication were randomly selected, and the plant height was measured. Four fully mature leaves were separated from each plant (16 leaves per treatment) to measure the leaf area, and their average was measured by a leaf area meter. Then, select samples were taken, and their fresh weight was recorded. The samples were placed in an oven at 70 °C for 48 h, and then their dry weight was recorded.

#### 4.4. Measurement of Leaf Mineral Elements

The leaf samples were subjected to mineralization following the AOAC [55] procedure. Initially, the dried samples were placed in a furnace and heated at 500 °C for 2 h, resulting in the formation of ash. The ash was then dissolved in 4 mL of diluted HNO<sub>3</sub> (1:1 ratio) with a density of 1.33. The solution was evaporated using a hot plate at 100 °C, leaving behind a residue. The residue was further heated at 500 °C for 1 h and allowed to cool. Subsequently, 10 mL of diluted HCl (1:1 ratio) with a density of 1.18 was added to the residue, and heat (100 °C) was applied on a hot plate to dissolve the residue. The concentration of phosphorus (P) in the resulting digest was measured using a spectrophotometer at a wavelength of 470 nm. Nitrogen (N) content was determined using the Kjeldahl method as described by AOAC [56]. Furthermore, the concentrations of potassium (K), calcium (Ca), magnesium (Mg), Mn, Fe, Zn, and Cu were measured using atomic absorption spectrometry (model 240FS, Agilent, Santa Clara, CA, USA), following the guidelines provided by AOAC [55]. Each treatment was replicated three times to ensure the accuracy and reliability of the results.

#### 4.5. Essential Oil Isolation

A total of 15 g of dried basil aerial parts were hydrodistilled for 3 h with a Clevenger-type apparatus. The essential oil obtained (three replications) was weighed with a digital scale with an accuracy of 0.0001 g and dehydrated with dry sodium sulfate. The essential oil percentage in each sample was calculated according to Equation (1). The yield of essential oil was obtained by multiplying the percentage of essential oil with the dry weight of the aerial parts (gr/plant) [57].

$$\text{Essential oil percentage\%} = (\text{Essential oil weight/dry weight of plant}) \times 100 \quad (1)$$

#### 4.6. GC/FID and GC/MS Analysis

The essential oils were subjected to analysis using gas chromatography (GC) in FID mode with a Thermo-UFM ultra-fast system (Agilent Scientific Instruments, USA). Separation was achieved using an HP-5 column (30 m length, 0.1 mm inner diameter, 0.40 µm film thickness). The carrier gas helium was utilized, and both the detector and injector (FID) were set to a temperature of 285 °C. The flow rate of the carrier gas was maintained at 1.1 mL/min, with a split ratio of 1:60. The oven temperature started at 60 °C for 5 min and then increased to 280 °C at a rate of 7 °C/min. The analysis was performed in triplicate.

For further characterization, a Varian 3400 GC/MS system was employed (Agilent Scientific Instruments, Santa Clara, CA, USA). A DB-5 fused silica column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) was used. The carrier gas helium was maintained at a flow rate of 1 mL/min, and the split ratio was set to 1:50. The transfer line temperature was set to 260 °C. Flame ionization detection was performed with a linear velocity of 31.5 cm/s and a voltage of 70 eV, covering a mass range of 50–450 amu. The oven temperature was programmed to increase from 50 to 240 °C at a rate of 4 °C/min [6].

The identification of essential oil components was accomplished by comparing their retention indices (RI) and mass spectra with those in Wiley, Adams, and NIST05 libraries, as well as references in the literature [58]. Additionally, co-injection with available authentic standards was utilized. The relative percentages of the essential oil constituents were determined by normalizing the peak areas obtained from GC-FID analysis [59,60].

#### 4.7. Anthocyanins

The Wagner [61] method was used to measure leaf anthocyanins. The leaf samples were thoroughly ground in a porcelain mortar containing an acidic methanol solution, and the obtained extract was placed in the dark for 24 h. After that, the samples were centrifuged, and the absorbance of the supernatant solution was recorded using a spectrophotometer at a wavelength of 550 nm; the content of anthocyanin was calculated in micromoles per g of fresh leaf weight.

#### 4.8. Preparation of Methanol Extract and Measurement of Total Phenolics and Flavonoids

A total of 1 g of the dried basil leaves (harvested in the early stages of flowering) was placed in a magnetic shaker with 25 mL of 80% methanol for 3 h. The samples were then centrifuged at 3000 rpm for 5 min, and the supernatant solution was collected to measure its antioxidant properties [62].

The content of total phenolics in the extracts was measured based on the Folin–Ciocâlteu method [63]: 100 mL of the extract with a concentration of 1 mg/mL was added to 500 mL of Folin's reagent. After 1 min, 1.5 mL of sodium bicarbonate 20% was added to each tube and then vortexed and incubated for 120 min at room temperature. The absorbance of the sample at 760 nm was read with a spectroscopic device. The standard curve was prepared by solutions of 50 to 500 mg/L of gallic acid in methanol. Total phenolic content was expressed as gallic acid equivalent (mg of gallic acid/g dry weight), which is a reference compound for determining phenolic content.

The flavonoid content was assessed using the aluminum chloride colorimetric method described by Chang et al. [64]. A total of 0.5 mL of each extract was combined with



1.5 mL of methanol. To this mixture, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were added. The resulting solution was incubated at room temperature for 30 min, and its absorbance at 415 nm was read by a spectrophotometer. Different concentrations of quercetin, 12.5–100 µg/mL in methanol, were used to draw a standard curve, and the dry extract content was expressed. Flavonoids were expressed as quercetin equivalent/g dry weight.

#### 4.9. Evaluation of Antioxidant Properties

##### 4.9.1. Free Radical Inhibition (DPPH)

In this method, 3.9 mL of a prepared DPPH (2,2-diphenyl-1-picrylhydrazyl) stock solution was added to a test tube. The DPPH stock solution was prepared by dissolving 0.004 g of DPPH in 100 mL of methanol. Then, 0.1 mL of each extract was added to the test tube containing the DPPH solution. The test tube was placed in a dark environment for 30 min. The absorbance of the solution was read at a wavelength of 517 nm. DPPH radical inhibition percentage was calculated using Equation (2). Then the results were expressed as IC<sub>50</sub> (an amount of antioxidant that is necessary for the concentration of DPPH to reach 50% of the initial value) [65].

$$I\% = [(A_0 - A_s)/A_0] * 100 \quad (2)$$

where I% represents free radical inhibition percentage, A<sub>0</sub> represents control absorption, and A<sub>s</sub> represents sample absorption.

##### 4.9.2. Ferric Reducing Antioxidant Potential (FRAP)

Antioxidants that have the ability to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup> and change the colorless TPTZ-Fe<sup>3+</sup> complex to the blue TPTZ-Fe<sup>2+</sup> complex, whose intensity can be measured at the wavelength of 593 nm. For this purpose, the concentration of 250 µg/mL of plant extract was taken and added to the final volume of 2 mL of FRAP solution containing TPTZ 10 mM (in 40 mM HCl), 20 mM iron chloride, and 300 mM acetate buffer at pH = 3.6. The above sample was placed at a temperature of 37 °C for 10 min, and the intensity of the resulting color was read at a wavelength of 593 nm against a blank. To draw the standard curve for the FRAP method, iron sulfate (FeSO<sub>4</sub> · 7H<sub>2</sub>O) with concentrations of 1000, 500, 250, and 125 µM was used, and the antioxidant capacity of the extracts was expressed as micromoles of Fe<sup>2+</sup> per gram of fresh weight of plant [66].

#### 4.10. Statistical Calculations

The mean of data obtained from the 2-year research was subjected to variance analysis according to the compound analysis, and the comparison of means was conducted with the LSD test at  $p \leq 0.05$ . Excel was used in order to draw graphs.

## 5. Conclusions

In the current study, basil plants grown in a hydroponic system had higher yields, which was due to the continuous supply of nutrients such as K, Ca, Mn, N, P, Mn, Fe, B, and Zn in this system. Field-grown basil plants had more woody growth and showed a higher stem dry weight. They also had higher levels of essential oil, phenolics, flavonoids, anthocyanins, and antioxidant activity. Therefore, in terms of biological properties and the content of bioactive components, field cultivation of basil is more suitable than hydroponic cultivation. However, there was no significant difference in the yield of essential oil in field cultivation and hydroponic systems. The level of methyl chavicol, the dominant component of basil essential oil, was higher in hydroponic culture, while the percentage of 1,8-cineole in the essential oil was very low in this system. The highest levels of linalool, *epi*-α-cadinol, δ-cadinene, and spathulenol were found in the greenhouse soil culture. It can be stated that the essential oils of plants grown in the hydroponic system had a higher economic value due to the higher levels of methyl chavicol.

Foliar spraying of Cu, Mn, Zn, Fe, and B significantly increased the leaf dry weight and anthocyanin content of basil. However, foliar application of micronutrients in each of the cultivation systems led to different results. In field conditions, the highest levels of total phenolics, total flavonoids, and antioxidant capacity were observed following Zn or Mn foliar application. In hydroponic cultivation, foliar spraying of Zn or B led to the highest levels of antioxidant activity, phenolics, and flavonoids. In all three cultivation systems, foliar application of B, Mn, or Zn led to the highest percentage and yield of essential oil. It seems that the difference in the level of nutrients in the field soil, greenhouse soil, and nutrient solution in the hydroponic system is the reason for the different responses of plants to the foliar application of elements in different cultivation substrates.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants13172498/s1>, Table S1: Variance analysis of the effect of foliar spraying of some micronutrient and cultivation system on biomass and phytochemical characteristics of basil; Table S2: Variance analysis of the effect of cultivation system and foliar spraying of some micronutrient on the amount of micro and macro elements in basil leaves; Table S3: Variance analysis of the effect of cultivation system and foliar spraying of some micronutrient on essential oil constituents of basil.

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**Data Availability Statement:** The datasets used and/or analyzed in this study are available from the corresponding author upon reasonable request.

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## Article

# Pharmacognostic Evaluation, Chemical Characterization, and Antibacterial Activity of *Bassia indica* (Wight) A.J. Scott

Fayyaz Anjum <sup>1</sup>, Saad Touqeer <sup>2,3</sup>, Muhammad Younus Khan <sup>4</sup>, QurraUllAin Jamil <sup>5</sup>, Ayesha Rida <sup>1</sup>, Jafir Hussain Shirazi <sup>6</sup>, Syeda Abida Ejaz <sup>7</sup>, Hafiz Muhammad Attaullah <sup>7</sup>, Ghulam Sarwar <sup>8</sup>, Zaeem Hayat Khan <sup>9</sup>, Muhammad Asif Wazir <sup>4</sup>, Barizah Malik <sup>10</sup>, Mohammed Aufy <sup>11,\*</sup> and Shahid Muhammad Iqbal <sup>1,\*</sup>

<sup>1</sup> Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan; fayyaz.anjum@iub.edu.pk (F.A.)

<sup>2</sup> College of Pharmacy, Al Ain University, Abu Dhabi 112612, United Arab Emirates

<sup>3</sup> AAU Health and Biomedical Research Center, Al Ain University, Abu Dhabi 112612, United Arab Emirates

<sup>4</sup> Department of Pharmacognosy, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

<sup>5</sup> Department of Pharmacy Practice, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

<sup>6</sup> Department of Pharmaceutics, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

<sup>7</sup> Department of Pharmaceutical Chemistry, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

<sup>8</sup> Department of Botany, Faculty of Chemical and Biological Sciences, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

<sup>9</sup> Department of Pharmacy, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

<sup>10</sup> School of Biochemistry and Biotechnology, Quaid-e-Azam Campus, University of the Punjab, Lahore 54590, Pakistan

<sup>11</sup> Division of Pharmacology and Toxicology, University of Vienna, UZA II, Josef-Holaubek-Platz 2, A-1090 Vienna, Austria

\* Correspondence: mohammed.aufy@univie.ac.at (M.A.); shahid.iqbal@iub.edu.pk (S.M.I.)

**Abstract:** *Bassia indica* (Wight) A.J. Scott is an Indian origin plant with documented medicinal and nutritional value, but has not been fully characterized yet. The present study was designed to establish pharmacognostic standards for the proper identification of the *B. indica* plant and its chemical characterization. The plant was standardized with World Health Organization (WHO) standardization tools and chemically characterized by Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectroscopy (GC-MS) analysis. Antibacterial potential was assessed by the zone of inhibition and minimum inhibitory concentration (MIC), and molecular docking studies were also performed. Pharmacognostic evaluation established the macroscopic and microscopic parameters for the identification of whole plant and its powder. Physicochemical parameters were also set forth while quantitative phytochemical analysis showed that the ethyl acetate fraction had the highest quantity of phenols, flavonoids, and tannins. FTIR analysis showed several functional groups such as phenols, alkanes, and alcohols while 55 phytochemicals were identified in the GC-MS analysis of the crude fraction. The crude extract and other fractions showed marked antibacterial activity, while the ethyl acetate fraction showed the least MIC (1.95–31.25 mg/mL). Phytochemicals identified in the GC-MS showed good molecular docking interactions against the DNA gyrase subunit B of bacteria with binding energies ranging from −4.2 to −9.4 kcal/mol. The current study describes the pharmacognostic characterization and phytochemical profiling of *B. indica* and provides scientific evidence to support its use in infections.

**Keywords:** *Bassia indica*; microscopic; macroscopic; antibacterial; GC-MS

## 1. Introduction

Plants have been a major source of agents used for the management of various diseases across the world for millennia and are still of contemporary importance as 80% of people worldwide depend on herbal remedies in their primary healthcare system, and over 25% of prescribed medicines in developed countries are of plant origin [1,2]. Over the last few decades, an upsurge has been witnessed in the public acceptance of natural therapies, leading to the increased usage of herbal medicines and phytonutrients. In Europe, over 1300 medicinals are being used for various ailments, while 118 out of the top 150 prescription drugs in the USA are based on natural resources [3]. Due to the increased demand for medicinal plants and the continuous loss of natural habitat, medicinal plants are facing a serious challenge to their existence. An upsurge in the usage of herbal medicines, a decrease in natural sources, and less availability of genuine crude drugs are resulting in adulteration and substitution practices [4]. Furthermore, crude drugs are collected, stored, distributed, and sold in raw form without any special packaging, which may also lead to adulteration and substitution [5].

As the use of herbal products continues to grow globally, concerns regarding their safety and public health issues are also increasingly recognized. One survey showed that 27% of herbal products commercialized worldwide are adulterated. The growing evidence of poor quality and a lack of authenticity is causing deep concerns [6]. Therefore, the proper identification of plants, their standardization, and documentation is very important for the quality of drugs. Identification includes the physical, biological, chemical, and biochemical features of plant species [7]. The WHO has set various qualitative and quantitative parameters for the standardization and quality control of herbal medicines. In pharmacognostic standardization, crude drugs are properly identified, their quality and purity is confirmed, and possible adulteration is observed. Qualitative methods of standardization such as organoleptic evaluation, toxicity testing, and bioassays contribute to the correct identification of crude drugs, while physicochemical analysis is carried out for their quantification [8].

*Bassia indica* (Wight) A.J. Scott [Syn.: *Kochia indica* Wight] belongs to the family Amaranthaceae [9]. This family includes a large number of halophytes that are well-known for their therapeutic applications in traditional medical systems and are used for infected skin wounds, internal worms, fungal infections, renal and rheumatic disorders [10] as a cardiogenic [11], anti-inflammatory, and antioxidant [12]. *B. indica* is an annual herb found in Egypt, India, Pakistan, Northern Africa, Saudi Arabia, and other countries [13]. The plant extracts are reported to have therapeutically important metabolites including polyphenols, sterols, alkaloids, glycosides, tannins, coumarins, terpenes, and saponins [14]. Moreover, it has been reported that this plant exhibits anti-inflammatory, anticancer [15], antioxidant [16], antimicrobial [17], antifungal [18], antiparasitic [14], anti-ulcer [12], and neuroprotective activities [19].

Despite the medicinal potential of *B. indica*, its pharmacognostic evaluation has not been performed yet. Therefore, the present study was designed to establish a pharmacognostic standard for the proper identification and purity of the plant to avoid adulteration. Along with this, comprehensive chemical characterization was carried out and the antibacterial activity was tested. Further in silico molecular docking was performed to identify the compounds responsible for the antibacterial effects. In vitro cytotoxicity and acute toxicity studies in mice were also conducted to establish its safety profile.

## 2. Results

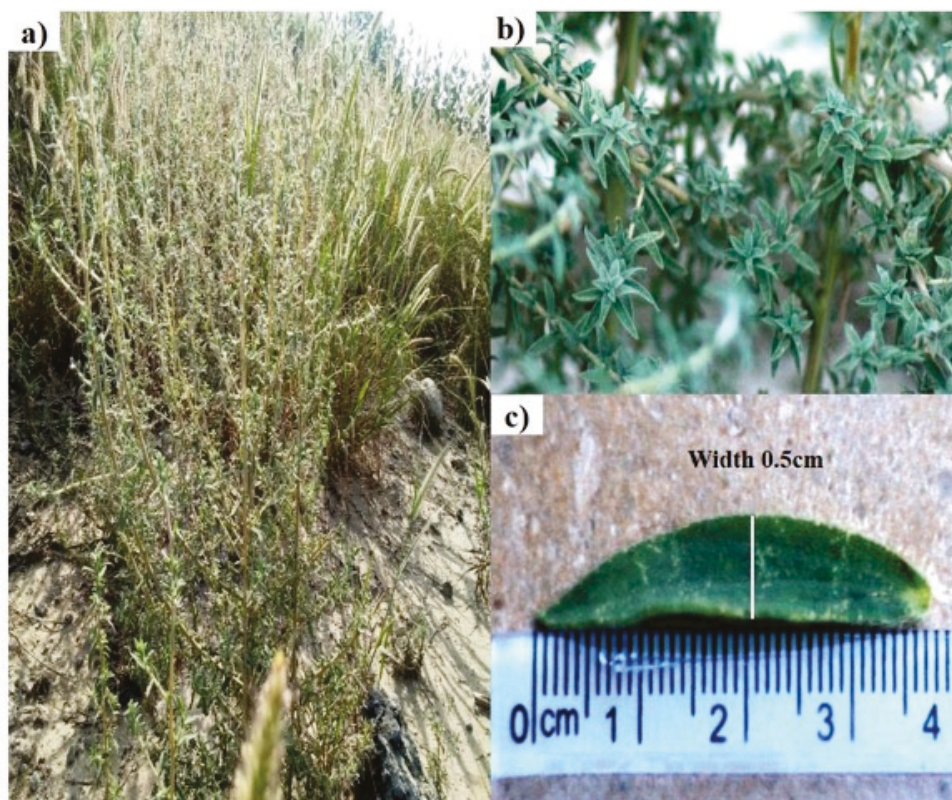
### 2.1. Extract Preparation and Fractionation

A total of 264 g *B. indica* extract was obtained from 3 kg powder of the aerial parts with an 8.8% yield. During fractionation, the highest yield (77%) was observed in the aqueous fraction while the least yield (1%) was observed in the *n*-hexane fraction.

## 2.2. Pharmacognostic Evaluation

### 2.2.1. Macroscopic Analysis

The fresh leaves of the plant were hairy, dark green in color, and decussate in arrangement with lanceolate tips (Figure 1b), which turned into light brown on drying. The leaves were 3.7 cm long and had a 0.5 cm width (Figure 1c). The stem was yellowish green and flowers were inconspicuous. The plant has a characteristic odor and bitter taste.

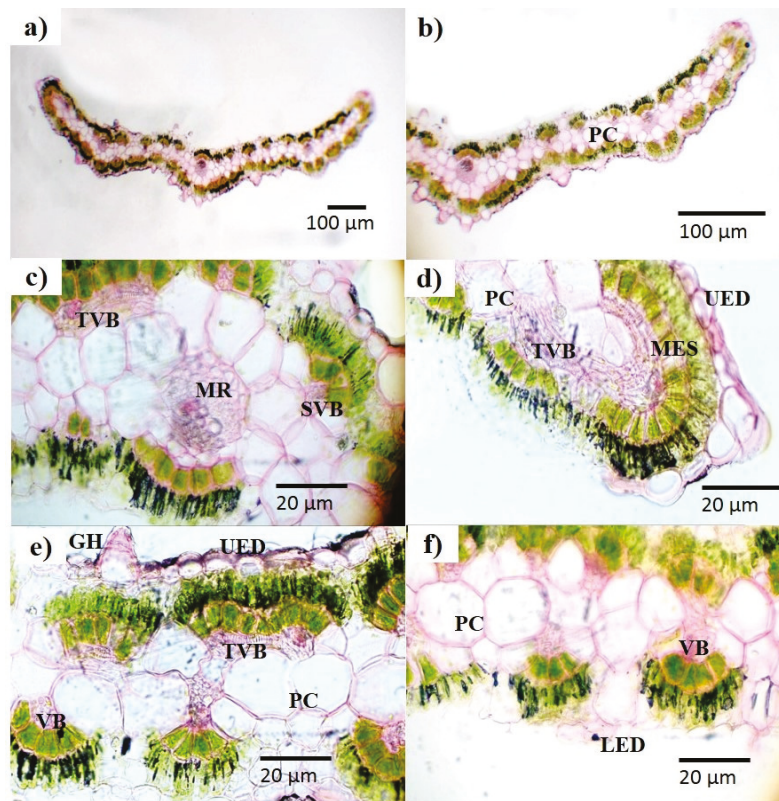


**Figure 1.** Different parts of the *B. indica* plant: (a) whole plant with area of growing; (b) plant stem and leaves showing color and summity; (c) leaf indicating length and width.

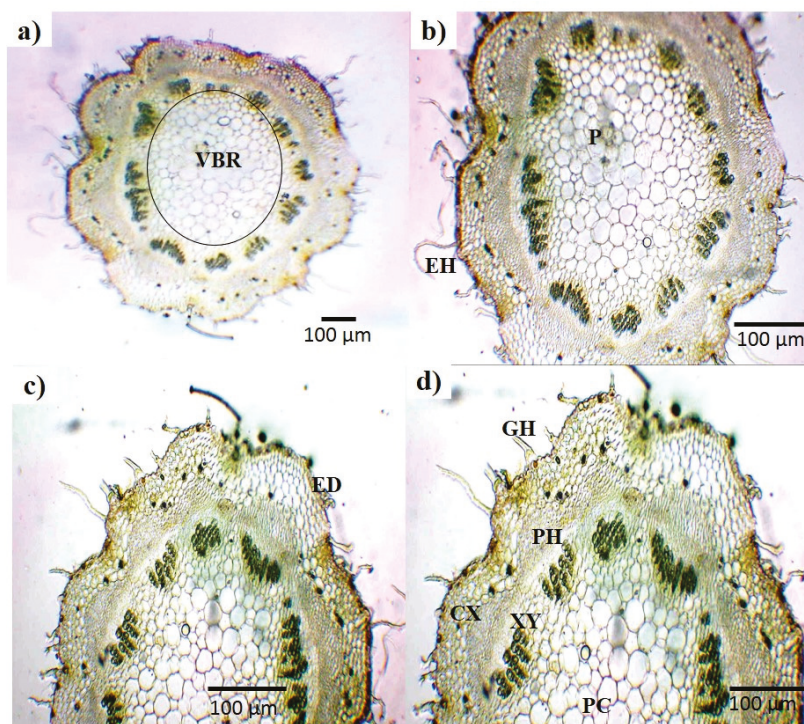
### 2.2.2. Microscopic Analysis

In the microscopic analysis of fresh leaves, complete transverse sections (TSs) of a leaf are presented (Figure 2a). In the center of the TS, a closely packed cell area (midrib) was seen, along with a number of whitish parenchyma cells in the adjacent areas (Figure 2b,c). Between the parenchyma cells and mesophyll, vascular bundles (cylindrical shaped) could be seen (Figure 2c,d). The TS of the leaf showed that an elongated cell layer covered both sides of the leaf called the upper and lower epidermal cells (Figure 2d,f). Glandular hair and mesophyll was also seen in the TS (Figure 2e). Similarly, the complete TS of the *B. indica* stem with a vascular bundle ring in the middle is presented (Figure 3a). A small and closely packed area, called the pith, and green colored xylem could also be visualized (Figure 3b,c). These different parts and cells of the leaf and stem were also observed in the dried powder of the aerial parts of *B. indica* (Figure 4). In addition to these structures, a number of stomata cells were seen in the epidermal cells along with abundant starch grains (Figure 4a,c). Microscopic analysis also showed that the powder had pitted as well as spiral vessels (Figure 4h,j).



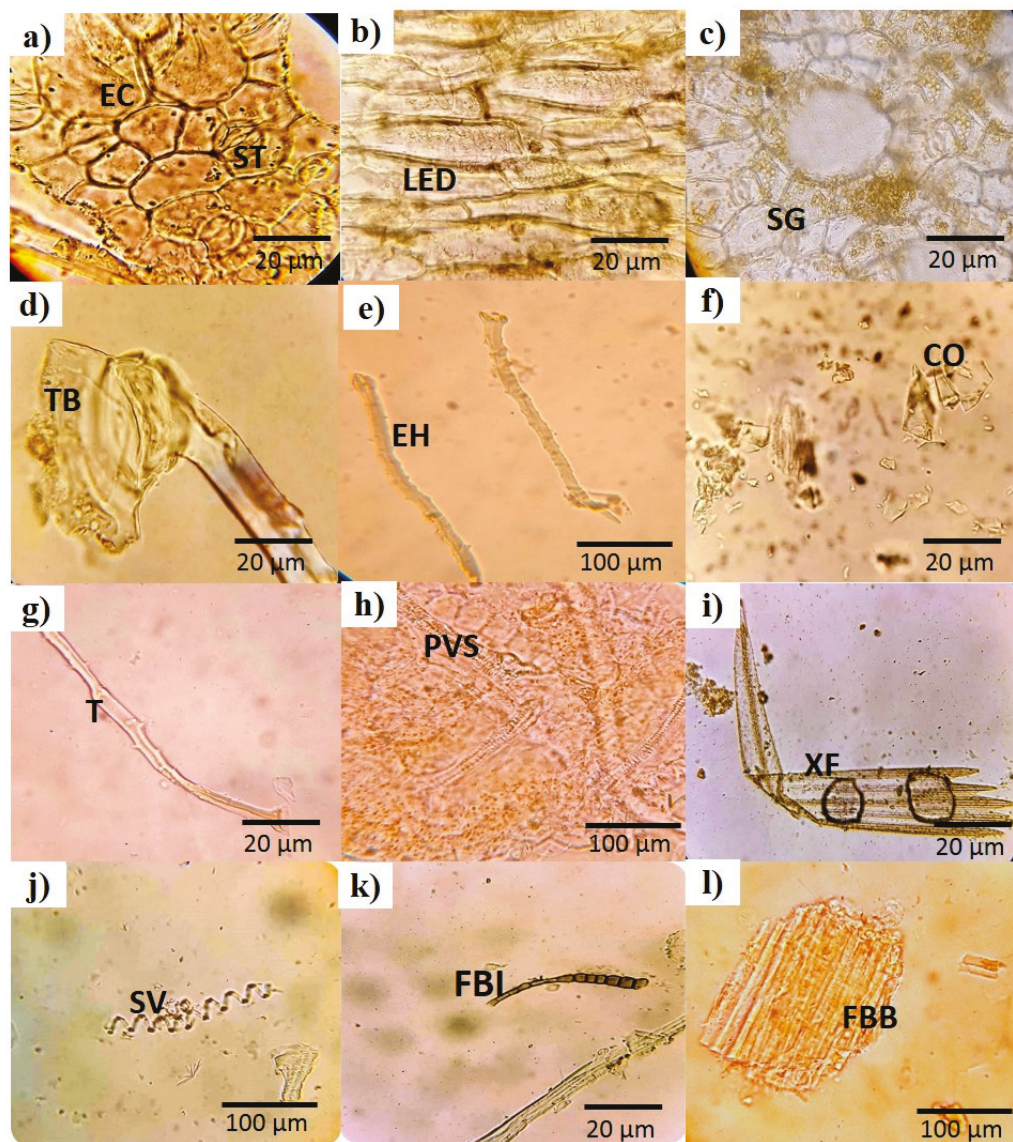


**Figure 2.** Different portions showing various tissues: (a) overall outline of leaf cross section (4×); (b) portion of same under 10×; (c) midrib portion; (d) leaf tip; (e) part with upper epidermis; (f) part with lower epidermis (40×); GH: glandular hair; LED: lower epidermis; MR: midrib; MES: mesophyll; PC: parenchyma; SVB: small vascular bundle; TVB: transverse vascular bundle; UED: upper epidermis; VB: vascular bundle.



**Figure 3.** Different portions showing various tissues: (a) overall outline of stem cross section (4×); (b) portion of same under 10×; (c) overall showing epidermis; (d) different parts including glandular

hair, cortex, phloem, xylem and parenchyma cells; CX: cortex, ED: epidermis, GH: glandular hairs, P: pith, PC: parenchyma cells, PH: phloem, VBR: vascular bundle ring, XY: xylem.



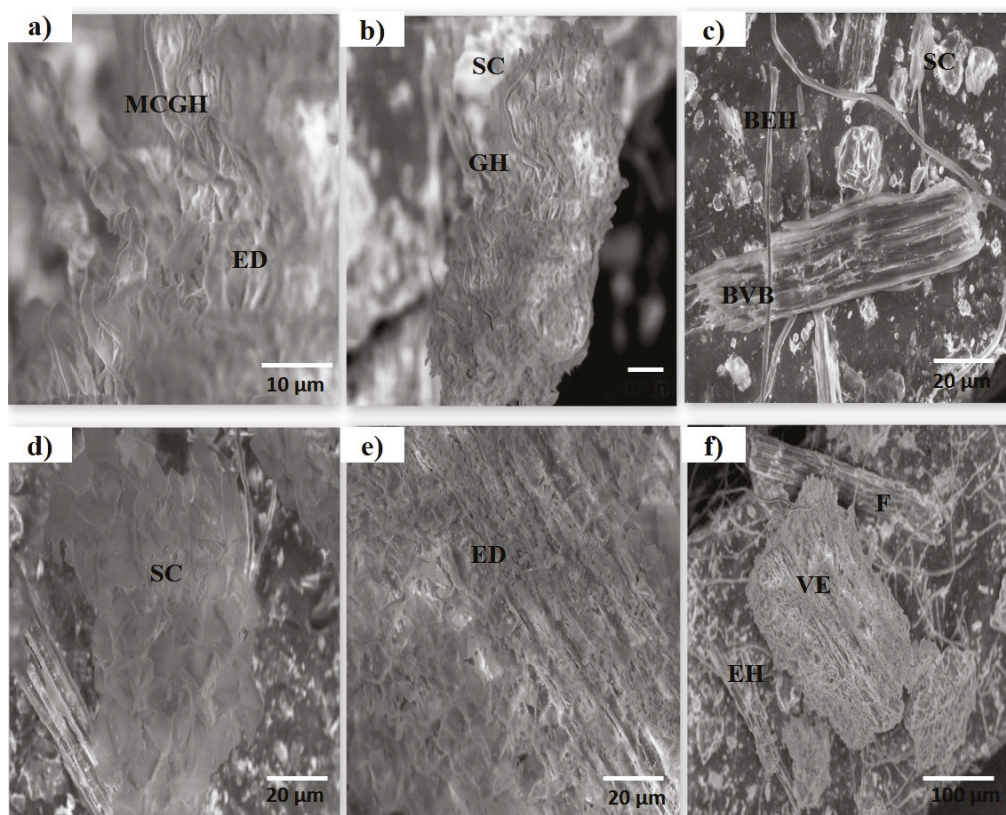
**Figure 4.** Powder microscopy showing different structures at 10× (e–h,j–l) and 40× (a–d,i). (a) epidermal cells (upper) with stomata; (b) lower epidermis cells; (c) cortical cells with starch grains; (d) trichome with base cell; (e) epidermal hair; (f) calcium oxalate crystals of different shape; (g) trichome; (h) pitted vascular system; (i) group of xylem fibers; (j) spiral thickening of vessels; (k,l): fiber bundle; CO: calcium oxalate crystals, FBI: intact fiber bundle, FBB: broken fiber bundle EH: epidermal hair, LED: lower epidermis, XF: xylem fibers, EC: epidermal cells, ST: stomata, SG: starch grain, SV: spiral thickening (lignin) of broken vessel, TB: trichome base, T: trichome, PVS: pitted vessel system.

### 2.2.3. Scanning Electron Microscopy

*B. indica* powder was observed under a scanning electron microscope for detailed anatomy. SEM analysis of the aerial parts (powder) showed the presence of epidermal and stomata cells embedded in fibers like a vascular bundle (Figure 5a). Many salt crystals and broken bundles of vessels were also seen (Figure 5b–d). Under the scanning electron



microscope, epidermal hairs and fibers were seen, which can be an important parameter for plant identification (Figure 5f).



**Figure 5.** Different fields of view of scanning electron microscopy: (a) segment of epidermis with multicellular glandular hair and epidermal cells (600×); (b) portion of same as (a) under 250×; (c) powder showing broken vascular bundle, salt crystals and broken epidermal hairs; (d) powder showing salt crystals and broken fiber cells; (e) epidermis showing tightly packed cells; (f) segments of vessel elements, fibers and hairs (60×) BEH: broken epidermal hairs, BVB: broken vascular bundle, EC: epidermal cells, ED: epidermis, EH: epidermal hair, F: fibers, GH: glandular hair, MCGH: multicellular glandular hairs, SC: salt crystals, VE: vessel elements.

#### 2.2.4. Extractive Value and Swelling Index

Percent extractive values of the *B. indica* powder was carried out in solvents with different polarities. The maximum extractive value was observed in the aqueous solvent (10.5%) and the minimum for *n*-hexane (Table 1). The swelling index of the plant powder was 0.6 cm.

**Table 1.** Extractive value of *B. indica*.

| Solvent           | Value (%) |
|-------------------|-----------|
| Distilled water   | 10.5      |
| <i>n</i> -Butanol | 3         |
| Ethyl acetate     | 2         |
| Dichloromethane   | 1.5       |
| <i>n</i> -Hexane  | 1         |

#### 2.2.5. Nutritional and Elemental Analysis

The nutritional analysis of the powder showed the presence of the highest amount of crude fibers (24.21%), followed by carbohydrates and proteins (Table 2). The total caloric value was 111.43 kcal/100 g of dry powder. The atomic absorption spectroscopy

of the *B. indica* extract showed the presence of different elements (Table 2), where the analysis showed the highest amount of calcium (470 ppm), followed by iron (161 ppm) and magnesium (55.6 ppm). The content of heavy metals (i.e., lead, cadmium, chromium, copper, and zinc) was less than the WHO maximum permissible element values.

**Table 2.** Nutritional and elemental analysis of *B. indica*.

| Parameter            | Value               |
|----------------------|---------------------|
| Nutritional analysis | (%)                 |
| Crude protein        | 12.39               |
| Crude fat            | 1.10                |
| Crude fiber          | 24.21               |
| Carbohydrate         | 16.61               |
| Ash value            | 14.52               |
| Moisture content     | 31.17               |
| Caloric value        | 111.43 (kcal/100 g) |
| Elemental content    | (mg/kg)             |
| Calcium              | 470                 |
| Cadmium              | 0.1                 |
| Chromium             | 0.05                |
| Copper               | 34.5                |
| Iron                 | 161                 |
| Magnesium            | 55.6                |
| Manganese            | 5.5                 |

#### 2.2.6. Fluorescence Analysis

Fluorescence analysis of the powdered aerial parts of *B. indica* was carried out with various reagents that showed different shades of colors such as light and dark brown, light greenish, black greenish, and purple (Table 3). Fluorescence analysis is an important parameter adopted to confirm the quality of the crude drug as different secondary metabolites present in the crude material exhibit different fluorescence under visible and ultraviolet (UV) light when treated with different reagents.

**Table 3.** Fluorescence analysis of the dried powder of the *B. indica* aerial parts.

| Reagents                             | Daylight       | UV 254 nm      |
|--------------------------------------|----------------|----------------|
| Powder as such                       | Greenish brown | Brown          |
| Methanol                             | Brown          | Dark brown     |
| Ethyl acetate                        | Light green    | Dark green     |
| Dichloromethane                      | Dark green     | Blue           |
| Chloroform                           | Brown          | Dark brown     |
| <i>n</i> -Butanol                    | Dark brown     | Blue           |
| <i>n</i> -Hexane                     | Dark green     | Blackish green |
| 50% HNO <sub>3</sub>                 | Green          | Pink           |
| Picric acids                         | Yellowish      | Brown          |
| 50% HCl                              | Green          | Light pink     |
| Conc. H <sub>2</sub> SO <sub>4</sub> | Black          | Dark brown     |
| 1% NaOH                              | Green          | Dark green     |
| Iodine                               | Light brown    | Blue           |
| 5% FeCl <sub>3</sub>                 | Yellow         | Dark brown     |

#### 2.2.7. Thin Layer Chromatography

In the TLC profile, different fluorescent compounds were separated depending upon their affinity toward the mobile phase polarity. Brown spots were observed when TLC plates were seen under UV light at 254 nm. The maximum number (5) of spots were seen when the chloroform and methanol (4:1) mobile phase was used. The appearance of multiple spots on the TLC plates suggests the presence of secondary metabolites like

flavonoids, phenolic compounds, glycosides, and saponins. The Rf values of different spots are listed in Table 4.

**Table 4.** Rf values of the *B. indica* extracts in different mobile phases.

| Extract           | Solvent    | Ratio    | Rf Value                     |
|-------------------|------------|----------|------------------------------|
| <i>n</i> -Hexane  | H:E.A      | 1:1      | 0.34, 0.55, 0.67, 0.85       |
|                   | H:E.A      | 3:1      | 0.15                         |
| Dichloromethane   | CH:M       | 4:1      | 0.50, 0.62, 0.70, 0.90       |
|                   | H:E.A      | 1:1      | 0.28, 0.52                   |
| Ethyl acetate     | H:E.A      | 1:1      | 0.544, 0.68, 0.85            |
|                   | CH:M       | 4:1      | 0.11, 0.26, 0.34             |
| <i>n</i> -Butanol | CH:M       | 4:1      | 0.10, 0.16, 0.51, 0.63, 0.85 |
|                   | H:E.A      | 1:1      | 0.23, 0.50, 0.59, 0.66       |
| Aqueous           | CH:M       | 4:1      | 0.88                         |
| Crude             | E.A:B:AA:W | 16:2:1:1 | 0.08, 0.94                   |

H: *n*-hexane, E.A: ethyl acetate, CH: chloroform, M: methanol, B: *n*-butanol, AA: acetic acid, W: water.

### 2.3. Phytochemical Analysis

#### 2.3.1. Qualitative Analysis

The presence of different important bioactive secondary metabolites was detected by phytochemical analysis. The appearance of different colors and precipitates confirmed the presence of several secondary metabolites including alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, and tannins.

#### 2.3.2. Quantitative Analysis of Total Phenols, Tannins, Flavonoids, and Saponins

Therapeutically important phytoconstituents like phenols, flavonoids, and tannins present in the extract were further quantified (Table 5). The quantitative analysis showed that the ethyl acetate fraction contained the highest amount of total phenols ( $419.10 \pm 11.76$ ), tannins ( $249.60 \pm 10.50$ ) mg tannic acid equivalent per gram of dried extract ( $\text{mg TAEg}^{-1}$ ), and flavonoids  $161.790 \pm 1.375$  mg quercetin equivalent per gram of dried extract ( $\text{mg QEg}^{-1}$ ). The saponin contents were greater in the aqueous fraction ( $40.5 \pm 0.9\%$ ) compared to the crude extract ( $30.9 \pm 2.2\%$ ). Significant amounts of these secondary metabolites present in the plant suggest that *B. indica* may have therapeutic potential.

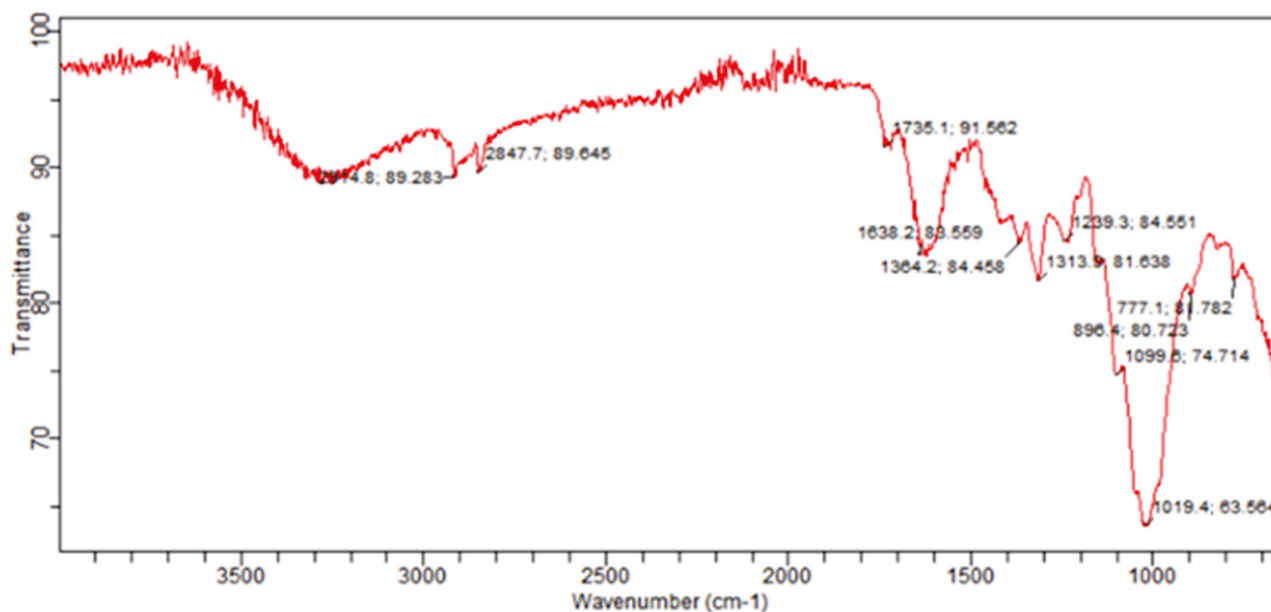
**Table 5.** Quantitative analysis of phenols, flavonoids, and tannins in the *B. indica* extracts.

| Fraction          | TPC ( $\text{mg TAEg}^{-1}$ ) | TTC ( $\text{mg TAEg}^{-1}$ ) | TFC ( $\text{mg QEg}^{-1}$ ) |
|-------------------|-------------------------------|-------------------------------|------------------------------|
| <i>n</i> -Hexane  | $36.00 \pm 2.9$               | $3.99 \pm 0.48$               | $24.089 \pm 3.952$           |
| Dichloromethane   | $303.48 \pm 25.28$            | $190.95 \pm 37.26$            | $106.223 \pm 3.437$          |
| Ethyl acetate     | $419.10 \pm 11.76$            | $249.60 \pm 10.50$            | $161.790 \pm 1.375$          |
| <i>n</i> -Butanol | $296.07 \pm 10.14$            | $156.97 \pm 29.47$            | $109.658 \pm 9.107$          |
| Aqueous           | $169.86 \pm 12.08$            | $104.17 \pm 26.93$            | $47.973 \pm 0.344$           |
| Crude             | $214.27 \pm 14.98$            | $107.50 \pm 30.27$            | $86.943 \pm 5.670$           |

TPC: total phenolic content, TTC: total tannin content, TFC: total flavonoid content.

### 2.4. FTIR Spectroscopy

The *B. indica* extract was also characterized with FTIR (Figure 6). The FTIR of the crude extract showed 11 characteristic peaks ranging from  $777.1$  to  $2914.8 \text{ cm}^{-1}$  in the frequency range and important medicinal functional groups (Table 6).



**Figure 6.** Fourier transform infrared spectrogram of *B. indica* showing different peaks.

**Table 6.** Functional groups and their wave numbers found in the FTIR of the *B. indica* powder.

| Wave Number (cm <sup>-1</sup> ) | Intensity of Estimation | Group or Functional Group Class    |
|---------------------------------|-------------------------|------------------------------------|
| 2914.8                          | S                       | O-H stretching (alcohol)           |
|                                 | M                       | C-H stretching (alkane)            |
| 2847.7                          | M                       | C-H stretching (alkane)            |
| 1735.1                          | W                       | C-H bending (aromatic compound)    |
| 1638.2                          | M                       | C=C stretching (conjugated alkene) |
| 1364.2                          | M                       | O-H bending (alcohol)              |
| 1313.9                          | S                       | S=O stretching (sulfone)           |
|                                 | M                       | O-H bending (phenol)               |
| 1239.3                          | S                       | C-O stretching (alkyl aryl ether)  |
|                                 | M                       | C-N stretching (amine)             |
| 1099.6                          | S                       | C-O stretching (secondary alcohol) |
| 1019.4                          | S                       | C=C bending (alkene)               |
| 896.4                           | M                       | C=C bend (alkenes like vanylidine) |
| 777.1                           | S                       | C-Cl stretching (halo compound)    |

S: strong, M: medium, W: weak.

## 2.5. GC-MS Analysis

GC-MS analysis of the *n*-butanol, ethyl acetate, and *n*-hexane fractions were performed and their chromatograms are shown in Figures 7–9, respectively. A total of 30 compounds were detected in the *n*-butanol fraction while 25 compounds were found in the ethyl acetate fraction (Tables 7 and 8). Six compounds were common among the *n*-butanol and ethyl acetate fractions including dodecane, 5,8-diethyl-17-pentatriacontene, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, 1,2-benzenedicarboxylic acid, diisooctyl ester, 24,25-dihydroxycholecalciferol, and 9,12,15-octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-. The total number of compounds detected in the *n*-hexane fraction was 62, out of which 14 compounds had a quality factor more than 80 (Table 9). One compound 10,13-octadecadiynoic acid, methyl ester was found to

be common among the *n*-butanol and *n*-hexane fractions. Similarly, the ethyl acetate and *n*-hexane fractions also contained isoaromadendrene epoxide as a common compound.

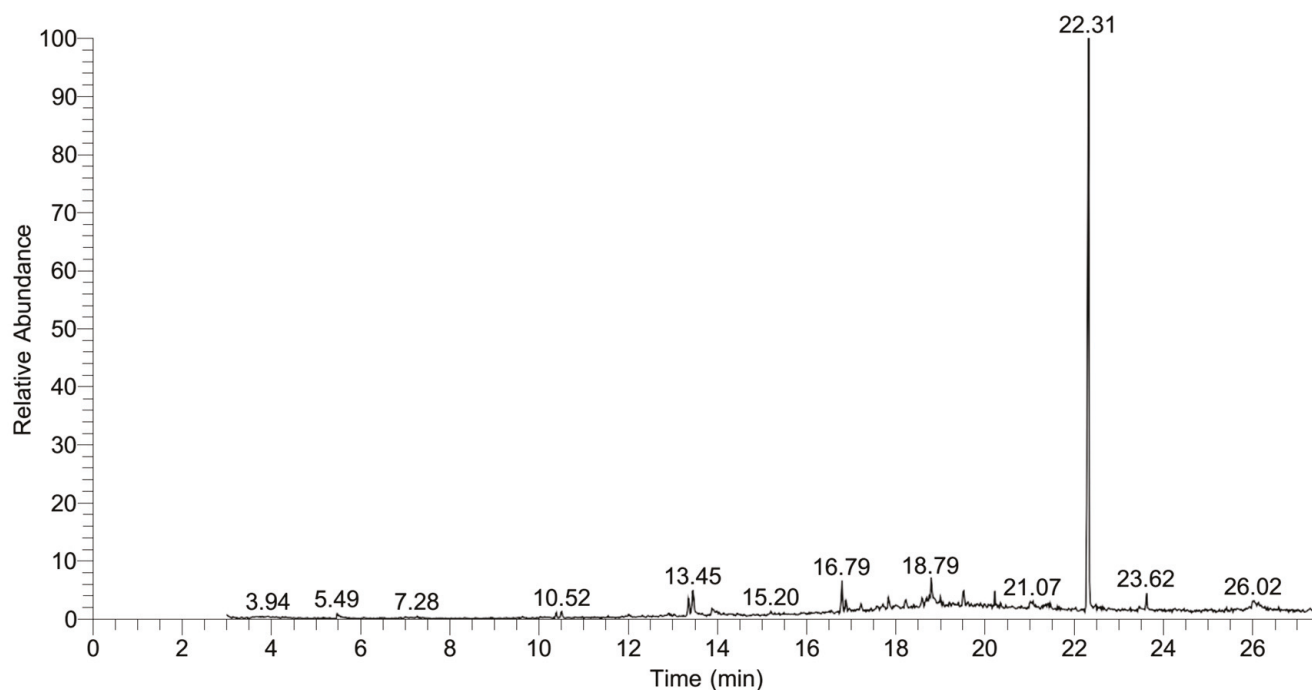


Figure 7. GC-MS chromatogram of the *n*-butanol fraction of the *B. indica* extract.

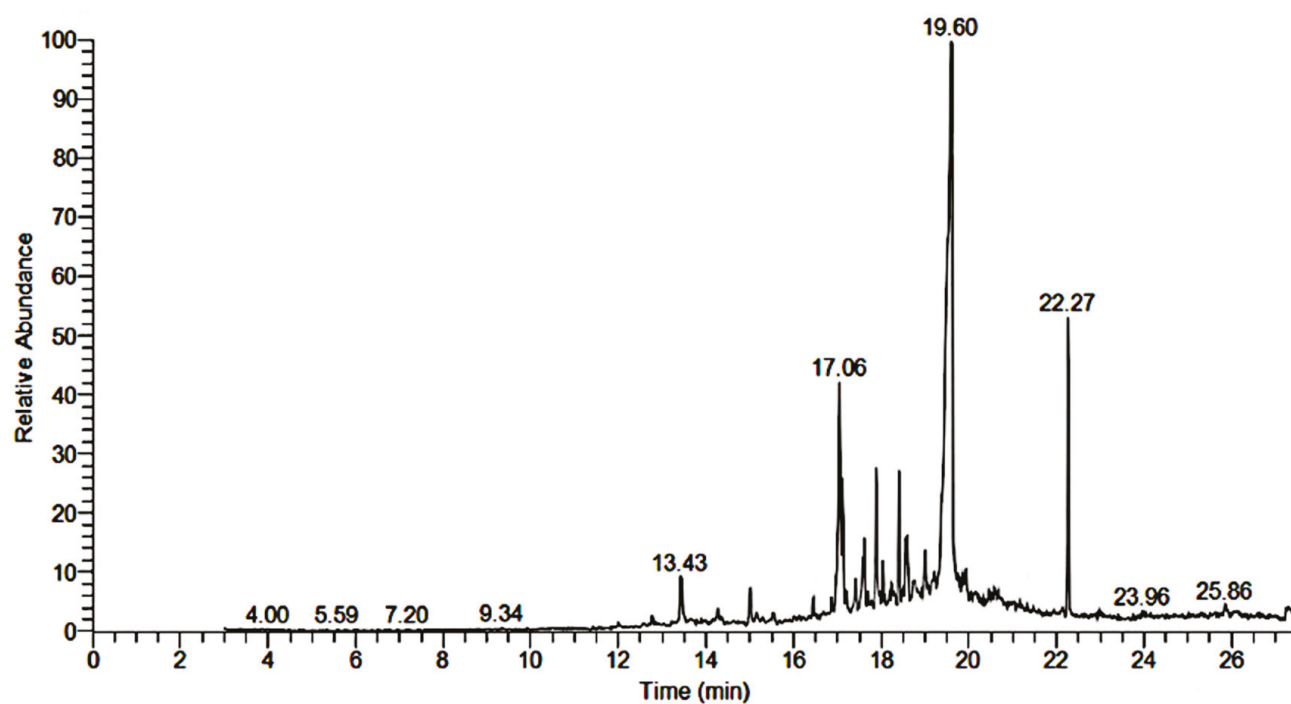


Figure 8. GC-MS chromatogram of the ethyl acetate fraction of the *B. indica* extract.



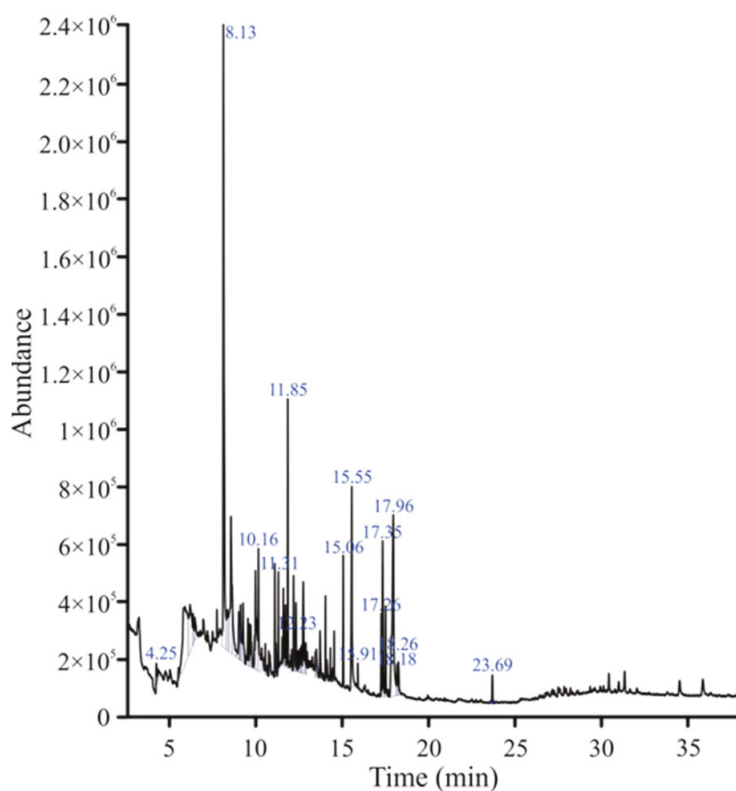


Figure 9. GC-MS chromatogram of the *n*-hexane fraction of the *B. indica* extract.

Table 7. Compounds identified in the GC-MS analysis of the *n*-butanol fraction of *B. indica*.

| Sr. NO | RT    | Area | Compound                                                              | Molecular Formula                                               | Molecular Weight |
|--------|-------|------|-----------------------------------------------------------------------|-----------------------------------------------------------------|------------------|
| NB1    | 3.92  | 1.10 | 6-Methoxy-2-phenyl-hexahydropyrano[2,3-b][1,3]dioxine-7,8 diol        | C <sub>14</sub> H <sub>18</sub> O <sub>6</sub>                  | 282.29           |
| NB2    | 4.28  | 0.17 | 2,5-Dihydro-5-methoxy-2-furanone                                      | C <sub>5</sub> H <sub>6</sub> O <sub>3</sub>                    | 114.10           |
| NB3    | 7.28  | 0.9  | 9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis    | C <sub>28</sub> H <sub>44</sub> O <sub>4</sub>                  | 444              |
| NB4    | 9.62  | 0.15 | Acetamide, N-methyl-N-[4-[2-acetoxymethyl-1-pyrrolidyl]-2-butynyl]-   | C <sub>14</sub> H <sub>22</sub> N <sub>2</sub> O <sub>3</sub>   | 266.33           |
| NB5    | 10.50 | 1.28 | Tetradecane                                                           | C <sub>14</sub> H <sub>30</sub>                                 | 198.38           |
| NB6    | 12.01 | 0.49 | Dodecane, 5,8-diethyl-                                                | C <sub>16</sub> H <sub>34</sub>                                 | 226.44           |
| NB7    | 12.31 | 0.20 | 2H-indeno[1,2-b]furan-2-one, 3,3a,4,5,6,7,8,8b-octahydro-8,8-dimethyl | C <sub>13</sub> H <sub>18</sub> O <sub>2</sub>                  | 206.28           |
| NB8    | 12.92 | 0.74 | Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester    | C <sub>16</sub> H <sub>30</sub> O <sub>4</sub>                  | 286.40           |
| NB9    | 13.43 | 5.20 | Hexadecane                                                            | C <sub>16</sub> H <sub>34</sub>                                 | 226.44           |
| NB10   | 13.92 | 3.16 | N-Acetyl-4-phenylbutylamine                                           | C <sub>12</sub> H <sub>17</sub> NO                              | 191              |
| NB11   | 15.20 | 0.77 | Myristyl monoethoxylate                                               | C <sub>16</sub> H <sub>34</sub> O <sub>2</sub>                  | 258.44           |
| NB12   | 15.92 | 0.15 | 2(3H)-Furanone, dihydro-5-tetradecyl                                  | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>                  | 282.46           |
| NB13   | 16.18 | 0.16 | trans-1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid                 | C <sub>14</sub> H <sub>22</sub> N <sub>2</sub> O <sub>8</sub>   | 346              |
| NB14   | 16.51 | 0.14 | 17-Pentatriacontene                                                   | C <sub>35</sub> H <sub>70</sub>                                 | 490.93           |
| NB15   | 16.81 | 3.39 | Bacteriochlorophyll-c-stearyl                                         | C <sub>52</sub> H <sub>72</sub> MgN <sub>4</sub> O <sub>4</sub> | 841.5            |
| NB16   | 17.20 | 0.77 | 2- Nonadecanone 2,4- dinitrophenylhydrazine                           | C <sub>25</sub> H <sub>42</sub> N <sub>4</sub> O <sub>4</sub>   | 462              |
| NB17   | 17.83 | 2.38 | 1-Docosanol                                                           | C <sub>22</sub> H <sub>46</sub> O                               | 326.6            |

Table 7. Cont.

| Sr. NO      | RT           | Area        | Compound                                                                                                                                                                                                                                        | Molecular Formula                                              | Molecular Weight |
|-------------|--------------|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|------------------|
| NB18        | 18.22        | 0.68        | 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione                                                                                                                                                                                       | C <sub>17</sub> H <sub>24</sub> O <sub>3</sub>                 | 276.37           |
| NB19        | 18.79        | 8.08        | Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester                                                                                                                                                                                       | C <sub>35</sub> H <sub>68</sub> O <sub>5</sub>                 | 568.91           |
| NB20        | 20.56        | 0.11        | 3-Pyridinecarboxylic acid, 2,7,10-tris(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-1,1,3,6,9-pentamethyl-4-oxo-4a,7a-epoxy-5H-cyclopenta[a]cyclo propa [f]cycloundecen-11-yl ester, [1aR(1aR*,2R*,3S*,4aR*,6S*,7S*,7aS*,8E,10R*,11R*,11aS*)]- | C <sub>32</sub> H <sub>39</sub> NO <sub>10</sub>               | 597.25           |
| NB21        | 21.05        | 1.77        | Dodecanecarboxamide, N-[2-(3-indolyl)ethyl]-                                                                                                                                                                                                    | C <sub>22</sub> H <sub>34</sub> N <sub>2</sub> O               | 342              |
| NB22        | 21.40        | 1.86        | Erucic acid                                                                                                                                                                                                                                     | C <sub>22</sub> H <sub>42</sub> O <sub>2</sub>                 | 338.57           |
| NB23        | 21.99        | 0.18        | 18,19-Secoyohimban-19-oic acid, 16,17,20,21-tetradecahydro-16-(hydroxy methyl)-, methyl ester, (15 $\alpha$ ,16E)-                                                                                                                              | C <sub>21</sub> H <sub>24</sub> N <sub>2</sub> O <sub>3</sub>  | 352              |
| NB24        | 22.31        | 48.18       | 1,2-Benzenedicarboxylic acid, diisooctyl ester                                                                                                                                                                                                  | C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>                 | 390              |
| NB25        | 23.62        | 2.08        | Squalene                                                                                                                                                                                                                                        | C <sub>30</sub> H <sub>50</sub>                                | 410              |
| <b>NB26</b> | <b>23.92</b> | <b>0.22</b> | <b>24,25-Dihydroxycholecalciferol</b>                                                                                                                                                                                                           | <b>C<sub>27</sub>H<sub>44</sub>O<sub>3</sub></b>               | <b>416</b>       |
| <b>NB27</b> | <b>24.29</b> | <b>0.76</b> | <b>Corynan-17-ol, 18,19-didehydro-10-methoxy-, acetate (ester)</b>                                                                                                                                                                              | <b>C<sub>22</sub>H<sub>28</sub>N<sub>2</sub>O<sub>3</sub></b>  | <b>368</b>       |
| NB28        | 25.0         | 0.54        | 9,12,15-Octadecatrenoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-                                                                                                                                                                | C <sub>27</sub> H <sub>52</sub> O <sub>4</sub> Si <sub>2</sub> | 496              |
| NB29        | 26.04        | 5.91        | Azafrin                                                                                                                                                                                                                                         | C <sub>27</sub> H <sub>38</sub> O <sub>4</sub>                 | 426              |
| NB30        | 27.8         | 0.13        | Butanoic acid,1a,2,5,5a,6,9,10,10a-octahydro-5,5a-di hydroxy-4-(hydroxymethyl)-1,1,7,9-tetra methyl-11-oxo-1H-2,8a-methanocyclop enta[a]cyclopropa[e]cyclodecen-6-yl ester                                                                      | C <sub>24</sub> H <sub>34</sub> O <sub>6</sub>                 | 418              |

Compounds written in bold characters were used for molecular docking; NB: *n*-butanol fraction, RT: retention time, \*: describe spatial configuration.

Table 8. Compounds identified in the GC-MS analysis of the ethyl acetate fraction of *B. indica*.

| Sr. No      | RT           | Area        | Compound                                                                                                            | Molecular Formula                                | Molecular Weight |
|-------------|--------------|-------------|---------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|------------------|
| EA1         | 12.0         | 0.15        | Octadecane, 3-ethyl-5-(2-ethylbutyl)-                                                                               | C <sub>26</sub> H <sub>54</sub>                  | 366.70           |
| EA2         | 12.80        | 0.59        | Illudol                                                                                                             | C <sub>15</sub> H <sub>24</sub> O <sub>3</sub>   | 252.35           |
| EA3         | 13.45        | 3.16        | Hexadecane                                                                                                          | C <sub>16</sub> H <sub>34</sub>                  | 226.44           |
| EA4         | 14.29        | 0.95        | 2-Naphthalenemethanol, decahydro- $\alpha,\alpha,4a$ -trimethyl-8-methylene-, [2R-(2 $\alpha,4a\alpha,8a\alpha$ )]- | C <sub>15</sub> H <sub>26</sub> O                | 222.36           |
| EA5         | 15.02        | 1.58        | Isoaromadendrene epoxide                                                                                            | C <sub>15</sub> H <sub>24</sub> O                | 220.35           |
| EA6         | 15.53        | 0.40        | Alpha-Santanol acetate                                                                                              | C <sub>17</sub> H <sub>26</sub> O <sub>2</sub>   | 262.38           |
| EA7         | 16.10        | 0.12        | 17-Pentatriacontene                                                                                                 | C <sub>35</sub> H <sub>70</sub>                  | 490.93           |
| EA8         | 16.44        | 0.46        | Acetate, [6-(acetyloxy)-5,5,8a-trimethyl-2-methyleneperhydro-1-naphthalenyl]methyl ester                            | C <sub>19</sub> H <sub>30</sub> O <sub>4</sub>   | 322.00           |
| EA9         | 17.06        | 12.11       | Bicyclo[2.2.2]octa-2,5-diene, 1,2,3,6-tetramethyl-                                                                  | C <sub>12</sub> H <sub>18</sub>                  | 162.27           |
| <b>EA10</b> | <b>17.61</b> | <b>2.97</b> | <b>5-Hydroxymethyl-1,1,4a-trimethyl-6-methylenedecahydronaphthalen-2-ol</b>                                         | <b>C<sub>15</sub>H<sub>26</sub>O<sub>2</sub></b> | <b>238.37</b>    |
| EA11        | 17.89        | 3.36        | Benzene, hexamethyl-                                                                                                | C <sub>12</sub> H <sub>18</sub>                  | 162.27           |
| <b>EA12</b> | <b>19.01</b> | <b>1.05</b> | <b>Gitoxigenin</b>                                                                                                  | <b>C<sub>23</sub>H<sub>34</sub>O<sub>5</sub></b> | <b>390.50</b>    |
| EA13        | 19.56        | 55.80       | 4,4-Dimethyladamantan-2-ol                                                                                          | C <sub>12</sub> H <sub>20</sub> O                | 180.29           |

Table 8. Cont.

| Sr. No | RT    | Area | Compound                                                                                                                                                                                                                                                                                                                                                      | Molecular Formula                                              | Molecular Weight |
|--------|-------|------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|------------------|
| EA14   | 20.17 | 0.11 | Androst-4-en-3-one, 9-fluoro-11,17-dihydroxy-17-methyl-, (11 $\alpha$ ,17 $\alpha$ )-                                                                                                                                                                                                                                                                         | C <sub>20</sub> H <sub>29</sub> FO <sub>3</sub>                | 336.40           |
| EA15   | 20.62 | 1.65 | <b>Methyl 3-(acetyloxy)-7,12-dioxocholan-24-oate</b>                                                                                                                                                                                                                                                                                                          | <b>C<sub>27</sub>H<sub>40</sub>O<sub>6</sub></b>               | <b>460.00</b>    |
| EA16   | 21.01 | 0.73 | <b>Agathic acid</b>                                                                                                                                                                                                                                                                                                                                           | <b>C<sub>20</sub>H<sub>30</sub>O<sub>4</sub></b>               | <b>334.40</b>    |
| EA17   | 22.27 | 5.58 | <b>1,2-Benzenedicarboxylic acid, diisooctyl ester</b>                                                                                                                                                                                                                                                                                                         | <b>C<sub>24</sub>H<sub>38</sub>O<sub>4</sub></b>               | <b>390.00</b>    |
| EA18   | 22.98 | 0.48 | <b>2,7-Diphenyl-1,6-dioxypyridazino[4,5:2',3']pyrrolo [4',5'-d]pyridazine</b>                                                                                                                                                                                                                                                                                 | <b>C<sub>20</sub>H<sub>13</sub>N<sub>5</sub>O<sub>2</sub></b>  | <b>355.30</b>    |
| EA19   | 23.98 | 0.67 | 9-Octadecene, 1-[2-(octadecyloxy)ethoxy]-                                                                                                                                                                                                                                                                                                                     | C <sub>38</sub> H <sub>76</sub> O <sub>2</sub>                 | 565.00           |
| EA20   | 24.43 | 0.17 | <b>5aH-3a,12-methano-1H-cyclopropa[5',6']cyclodeca [1',2':1,5]cyclopenta[1,2-d][1,3]dioxol-13-one, 1a,2,3,9,12,12a-hexahydro-9-hydroxy-10 -(hydroxymethyl)-1,1,3,5,7,7-hexamethyl-, [1aR-(1<math>\alpha</math>,3<math>\alpha</math>,3a<math>\alpha</math>,5a<math>\alpha</math>,8aR*,9<math>\alpha</math>,12<math>\alpha</math>,12a<math>\alpha</math>)]-</b> | <b>C<sub>23</sub>H<sub>32</sub>O<sub>5</sub></b>               | <b>388.50</b>    |
| EA21   | 24.43 | 0.17 | <b>Betulin</b>                                                                                                                                                                                                                                                                                                                                                | <b>C<sub>30</sub>H<sub>50</sub>O<sub>2</sub></b>               | <b>442.70</b>    |
| EA22   | 24.72 | 0.13 | <b>24,25-Dihydroxycholecalciferol</b>                                                                                                                                                                                                                                                                                                                         | <b>C<sub>27</sub>H<sub>44</sub>O<sub>3</sub></b>               | <b>416.00</b>    |
| EA23   | 25.86 | 2.47 | <b>Stigmasterol</b>                                                                                                                                                                                                                                                                                                                                           | <b>C<sub>29</sub>H<sub>48</sub>O</b>                           | <b>412.70</b>    |
| EA24   | 26.63 | 0.63 | 2,2,3,5,5-Pentachloro-7,7-bis(chloromethyl)-1 (dichloromethyl)bicyclo[2.2.1]heptane                                                                                                                                                                                                                                                                           | C <sub>10</sub> H <sub>9</sub> Cl <sub>9</sub>                 | 448.20           |
| EA25   | 27.30 | 0.68 | 9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-                                                                                                                                                                                                                                                                             | C <sub>27</sub> H <sub>52</sub> O <sub>4</sub> Si <sub>2</sub> | 496.90           |

Compounds written in bold characters were used for molecular docking; EA: ethyl acetate fraction, RT: retention time, \*: describe spatial configuration.

Table 9. Compounds identified in the GC-MS analysis of the *n*-hexane fraction of *B. indica*.

| Sr. No | RT    | Area  | Compound                                                     | Molecular Formula                                | Molecular Weight |
|--------|-------|-------|--------------------------------------------------------------|--------------------------------------------------|------------------|
| NH1    | 4.25  | 0.54  | <b>5-Methylfurfural</b>                                      | <b>C<sub>6</sub>H<sub>6</sub>O<sub>2</sub></b>   | <b>110.11</b>    |
| NH2    | 8.13  | 16.94 | <b>2-Methoxy-4-vinylphenol</b>                               | <b>C<sub>9</sub>H<sub>10</sub>O<sub>2</sub></b>  | <b>150.17</b>    |
| NH3    | 10.16 | 2.59  | <b>4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one</b> | <b>C<sub>13</sub>H<sub>18</sub>O</b>             | <b>190.28</b>    |
| NH4    | 11.31 | 2.99  | <b>Megastigmatrienone</b>                                    | <b>C<sub>13</sub>H<sub>18</sub>O</b>             | <b>190.28</b>    |
| NH5    | 12.23 | 0.65  | Ar-turmerone                                                 | C <sub>15</sub> H <sub>20</sub> O                | 216.31           |
| NH6    | 15.06 | 1.58  | Hexadecanoic acid, methyl ester                              | C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>   | 270.45           |
| NH7    | 15.25 | 4.41  | <i>n</i> -Hexadecanoic acid                                  | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>   | 256.42           |
| NH8    | 15.91 | 0.28  | Hexadecanoic acid, ethyl ester                               | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>   | 284.47           |
| NH9    | 17.26 | 1.09  | 9,12-Octadecadienoic acid, methyl ester                      | C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>   | 294.47           |
| NH10   | 17.35 | 2.91  | 9,12,15-Octadecatrienoic acid                                | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>   | 278.40           |
| NH11   | 17.96 | 8.58  | 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-                         | C <sub>18</sub> H <sub>32</sub> O                | 264.44           |
| NH12   | 18.18 | 0.59  | Linoleic acid ethyl ester                                    | C <sub>20</sub> H <sub>36</sub> O <sub>2</sub>   | 308.49           |
| NH13   | 18.26 | 0.89  | Ethyl Oleate                                                 | C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>   | 310.51           |
| NH14   | 23.69 | 0.49  | <b>1,2-Benzenedicarboxylic acid, monopentyl ester</b>        | <b>C<sub>13</sub>H<sub>16</sub>O<sub>4</sub></b> | <b>236.26</b>    |

Compounds written in bold characters were used for molecular docking; RT: retention time, NH: *n*-hexane fraction.

## 2.6. Acute Toxicity and Cytotoxicity

Oral acute toxicity was performed on mice and no mortality was observed. Furthermore, no sign of any toxicity like behavioral changes and any apparent injury or deformity was seen in animals throughout the study. Therefore, the extract was found to be safe up to a dose of 2000 mg/kg. The MTT cytotoxicity assay, carried out in mouse fibroblast cells, showed that *B. indica* is safe and did not cause any toxicity.

## 2.7. Antimicrobial Activity

### 2.7.1. Disc Diffusion Method

The antimicrobial assay of the crude extract and its *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol, and aqueous fractions showed good antibacterial activity against Gram-positive and Gram-negative bacteria. The zone of inhibition at 10, 30, and 100 mg/mL of all fractions was measured (Table 10). Results were dose dependent and the largest zone of inhibition (27 mm) was seen in the ethyl acetate fraction against *E. coli*. The aqueous extract was more effective at a lower dose than a higher dose; no growth inhibition of *B. subtilis* was seen at 100 mg/mL. The effects were comparable to the standard drug ceftriaxone (1 mg/mL); the fractions were more effective against *E. coli*, and the least effective against *P. aeruginosa*.

**Table 10.** Zone of inhibition of the *B. indica* extracts.

| Fractions         | Conc.   | <i>B. subtilis</i> | <i>S. aureus</i> | <i>P. aeruginosa</i> | <i>S. typhi</i> | <i>E. coli</i> |
|-------------------|---------|--------------------|------------------|----------------------|-----------------|----------------|
|                   | (mg/mL) | Mm                 |                  |                      |                 |                |
| <i>n</i> -Hexane  | 10      | 6                  | 10.5             | 8.5                  | 6               | 10.5           |
|                   | 30      | 12                 | 9                | 9                    | 8.5             | 15             |
|                   | 100     | 11.5               | 14               | 9.5                  | 9               | 12.5           |
| Dichloromethane   | 10      | 9                  | 7.5              | 7.5                  | 4.5             | 4              |
|                   | 30      | 9.5                | 11               | 8                    | 9               | 13             |
|                   | 100     | 13                 | 12               | 11.5                 | 10.5            | 13             |
| Ethyl acetate     | 10      | 10                 | 9.5              | 8.5                  | 8               | 5              |
|                   | 30      | 15.5               | 11.5             | 9                    | 9.5             | 8.5            |
|                   | 100     | 13                 | 14.5             | 10.5                 | 9.5             | 27             |
| <i>n</i> -Butanol | 10      | 9                  | 8                | 5                    | 9               | 9.5            |
|                   | 30      | 10.5               | 9                | 7.5                  | 4.5             | 11.5           |
|                   | 100     | 13                 | 10.5             | 11.5                 | 11              | 14             |
| Aqueous           | 10      | 7.5                | 9.5              | 8.5                  | 9.5             | 9.5            |
|                   | 30      | 8                  | 13.5             | 7                    | 7               | 5.5            |
|                   | 100     | nil                | 9.5              | 7.5                  | 6.5             | 9              |
| Crude             | 10      | 11                 | 8.5              | 7                    | 5               | 8.5            |
|                   | 30      | 11                 | 9                | 4.5                  | 8               | 10.5           |
|                   | 100     | 14.5               | 11               | 9                    | 8               | 12.5           |
| Ceftriaxone       | 1       | 15                 | 16               | 7.5                  | 11              | 15.5           |

### 2.7.2. Minimum Inhibitory Concentration

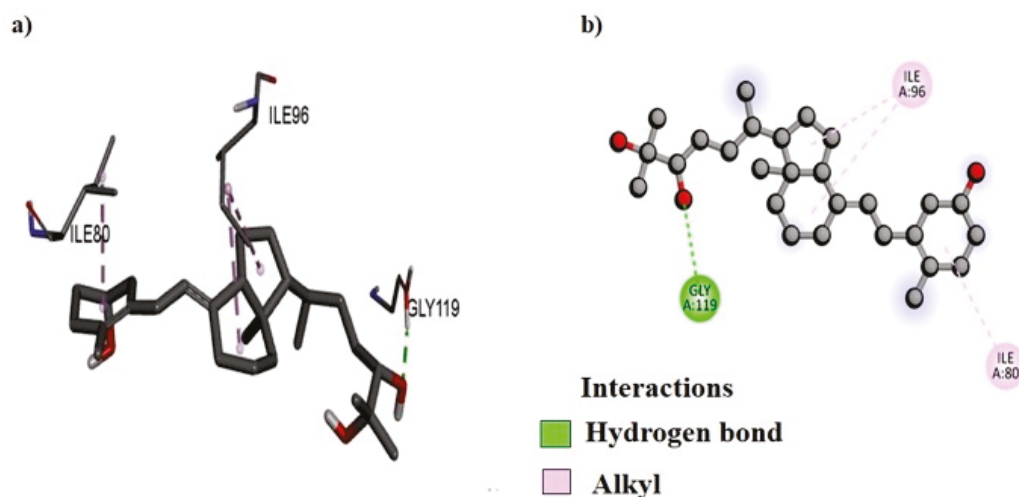
The MIC was also observed and the results indicated that the ethyl acetate fraction had the lowest minimum inhibitory concentration against all of the tested bacterial strains ranging from 1.95 to 31.25 mg/mL. The MIC was the lowest (1.95 mg/mL) against *B. subtilis* and highest (31.25 mg/mL) against *S. typhi*. The *n*-butanol fraction showed overall promising results, with the MIC ranging from 3.91 to 7.81 mg/mL. The *n*-hexane fraction was found to be the least effective (MIC > 100 mg/mL) against the tested strains, with zero response against *E. coli* (Table 11).

**Table 11.** Minimum inhibitory concentration of the *B. indica* extracts.

| Fractions         | <i>B. subtilis</i> | <i>S. aureus</i> | <i>P. aeruginosa</i> | <i>S. typhi</i> | <i>E. coli</i> |
|-------------------|--------------------|------------------|----------------------|-----------------|----------------|
|                   | mg/mL              |                  |                      |                 |                |
| <i>n</i> -Hexane  | >100               | >100             | >100                 | >100            | Nil            |
| Dichloromethane   | 3.91               | 7.81             | 3.91                 | 62.5            | 31.25          |
| Ethyl acetate     | 1.95               | 3.91             | 3.91                 | 7.81            | 3.91           |
| <i>n</i> -Butanol | 3.91               | 31.25            | 3.91                 | 31.25           | 3.91           |
| Aqueous           | 31.25              | 62.5             | 31.25                | 62.5            | 62.5           |
| Crude             | 31.25              | 31.25            | 31.25                | 62.5            | 62.5           |
| Ceftriaxone       | 1.95               | 3.91             | 0.98                 | 0.98            | 7.81           |

## 2.8. Molecular Docking

DNA gyrase was chosen as the molecular docking target for the *B. indica* extracts for several reasons. Firstly, DNA gyrase plays a crucial role in bacterial DNA replication and transcription, making it a potential target for antibacterial agents. Secondly, it is a well-validated target for existing antibacterial drugs, enhancing the likelihood of finding effective compounds in the extracts. Additionally, numerous studies have shown that plant-derived compounds can inhibit DNA gyrase [20], suggesting that *B. indica* may contain phytochemicals that target the DNA gyrase. Additionally, targeting DNA gyrase also offers the advantage of broad-spectrum antibacterial activity and can potentially complement other antibacterial mechanisms, leading to synergistic effects. These factors collectively justify the focus on DNA gyrase in identifying antibacterial compounds in *B. indica* [21]. Our analysis revealed that several amino acids were involved in forming both bonding and non-bonding interactions with the ligand. The docking protocol was validated by first separating the co-crystal ligand from the active pocket of the complex, and then re-docking was performed to validate its accuracy. The co-crystal ligand docking score and figures are given in the Supplementary Materials (Table S1; Figures S1–S9). The molecular docking scores of selected compounds of different fractions show that the best docked compound, NB23, from the *n*-butanol fraction bound with a  $-8.4$  kcal/mol binding energy (Table 12). The 2D and 3D binding interactions between NB23 and the active pocket of DNA gyrase subunit B of *P. aeruginosa* were also visualized (Figure 10). Amino acid residue GLY119 interacts via forming conventional hydrogen bonds while ILE96 and ILE80 interact by alkyl linkage. Similarly, the best docked compound, EA19, from the ethyl acetate fraction bound with a  $-9.4$  kcal/mol energy within the active pocket of DNA gyrase subunit B of *S. typhi* and its 2D and 3D binding interactions are presented in Figure 11. The docking analysis shows that amino acid residue GLN837 was found to have conventional hydrogen bond interactions with EA19 while ARG580 and ARG838 formed pi-cation, and the VAL787 and ILE736 amino acid residues interacted with EA19 by pi-sigma and pi-alkyl linkages (Figure 11). The binding interactions of NH4 with the active site of DNA gyrase from *P. aeruginosa*, both in two-dimensional (2D) and three-dimensional (3D) visuals are also presented in Figure 12. The amino acid GLY79 in the DNA gyrase established conventional hydrogen bonds with NH4. Furthermore, three other amino acids, ILE96, ILE80, and PRO81, formed alkyl interactions with NH4.

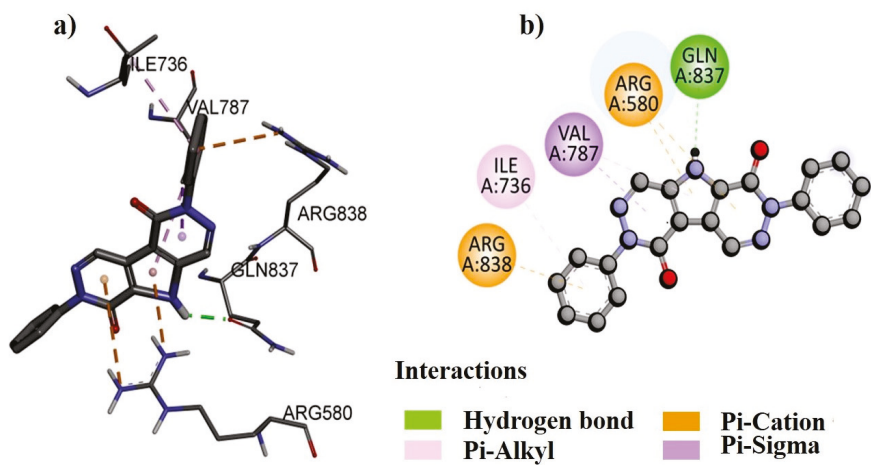


**Figure 10.** The predicted 3D (a) and 2D (b) binding modes of compound NB23 against the DNA gyrase subunit B of *P. aeruginosa*.

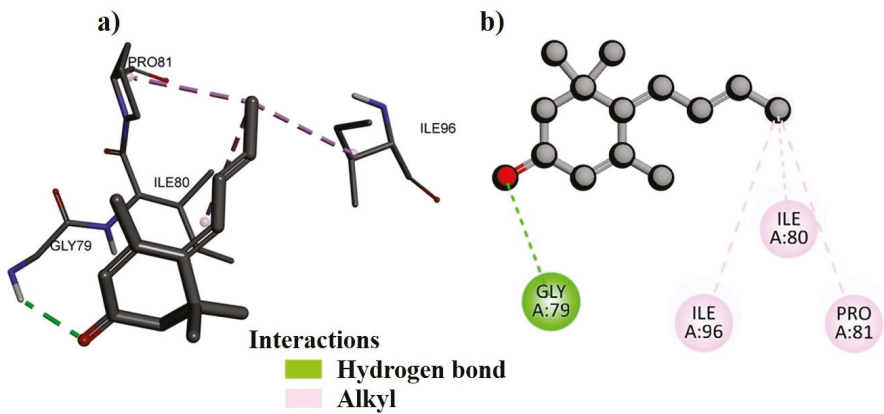


**Table 12.** Molecular docking scores of the *n*-butanol, ethyl acetate, and *n*-hexane fractions with the targeted proteins.

| Compounds | <i>B. subtilis</i> | <i>E. coli</i> | <i>P. aeruginosa</i> | <i>S. aureus</i> | <i>S. typhi</i> |
|-----------|--------------------|----------------|----------------------|------------------|-----------------|
|           | kcal/mol           |                |                      |                  |                 |
| NB1       | −6.6               | −7.3           | −8.0                 | −7.5             | −7.5            |
| NB7       | −5.7               | −7.4           | −7.4                 | −7.0             | −6.4            |
| NB26      | −7.2               | −6.6           | −8.4                 | −7.8             | −7.3            |
| NB27      | −6.3               | −6.5           | −7.8                 | −7.3             | −7.5            |
| EA10      | −5.7               | −5.4           | −6.3                 | −7.2             | −6.2            |
| EA12      | −7.5               | −7.5           | −8.4                 | −7.8             | −8.6            |
| EA15      | −6.6               | −7.1           | −8.8                 | −8.0             | −8.3            |
| EA16      | −6.6               | −7.1           | −7.6                 | −7.3             | −7.5            |
| EA17      | −4.2               | −4.4           | −5.3                 | −6.4             | −5.6            |
| EA18      | −8.2               | −8.5           | −9.1                 | −9.2             | −9.4            |
| EA20      | −7.5               | −7.4           | −8.7                 | −8.1             | −9.3            |
| EA21      | −7.4               | −6.6           | −7.5                 | −7.7             | −8.6            |
| EA22      | −6.8               | −6.0           | −7.3                 | −7.4             | −7.0            |
| EA23      | −7.5               | −7.1           | −8.3                 | −7.8             | −8.1            |
| NH1       | −4.2               | −4.5           | −4.8                 | −4.6             | −4.2            |
| NH2       | −4.8               | −5.5           | −5.9                 | −5.5             | −5.0            |
| NH3       | −5.5               | −5.9           | −6.4                 | −6.4             | −5.5            |
| NH4       | −5.8               | −6.1           | −6.8                 | −6.7             | −5.8            |
| NH14      | −5.0               | −6.2           | −6.4                 | −5.8             | −5.3            |



**Figure 11.** The predicted 3D (a) and 2D (b) binding modes of compound EA19 against the DNA gyrase subunit B of *S. typhi*.



**Figure 12.** The predicted 3D (a) and 2D (b) binding modes of compound NH4 against the DNA gyrase subunit B of *P. aeruginosa*.

### 3. Discussion

The standardization of medicinal plants is of great importance as it plays a legitimate role in understanding its botanical value, chemical structure, and clinical suitability; moreover, it may be helpful in finding morphologically similar and adulterated species [7,22]. The World Health Organization has laid down many standardization parameters that ensure the safety, efficacy, and quality of crude drugs [23]. The first and quickest method of drug identification and adulteration or purity is macroscopic evaluation. Macroscopic evaluation of the plant showed that it has green colored lanceolate leaves, a hairy stem, fibrous texture, and other characteristic parameters (Figure 1). These results allow for the easy identification of *B. indica* when harvesting from its natural habitat and preventing unintentional adulteration. These organoleptic characters are consistent with the previously reported description of the *B. indica* plant [24,25]. Microscopic evaluation parameters have an important role in the identification and validation of the plant. Anatomical histology and powder microscopy act as excellent tools for the authentication, adulteration, or purity of crude drugs [26]. Anatomical structures observed in the present microscopic study of the leaf, stem, and powder provide necessary information about the morphological characteristics of *B. indica*, which can aid in the identification and authentication of the plant material [27]. Similar structures have also been reported in other plants of the same genus [28,29]. Stomata observed in the epidermis prevent a cellular imbalance in plants; this adaptive strategy helps the plant to survive in drought conditions. Trichomes maintain still air on the leaves and are involved in secretory functions [30]. Scanning electron microscopy (SEM) was used to measure and describe the surface morphology of the plant material. Such morphological characteristics are important parameters in the identification of the sample [31]. Xylem observed in the SEM images had a narrow opening, which allows for the capillary movement of water and decreases its loss; the phloem ensures the storage of food. Therefore, the water conservation and food storage ability of different parts of plants help the plant survive in drought conditions. Similar anatomical features have also been observed in other plants growing in drought conditions [30,32,33]. Moreover, these observations provide information about the physical characteristics of the plant that may affect its solubility [28]. Extraction with different solvents showed the maximum yield in the aqueous solution. These results indicate that the plant material contains more polar compound (i.e., alkaloids, flavonoids, phenolics, saponins, and polysaccharides). It is reported that polar compounds often exhibit different biological activities such as antioxidant, anti-inflammatory, antimicrobial, and anticancer activities. A high aqueous extractive value indicates the presence of polar compounds, which imparts medicinal importance to plants [15,34]. Swelling of the plant material, when soaked in a solvent, indicates the presence of mucilage found in the crude drug. The swelling index of the powder observed in the current study was in line with previously reported studies performed on the other halophytic plant *Salsola kali*. Mucilages are mainly polysaccharides, and the swelling index properties are specific to their pharmaceutical utility [28]. The nutrients present in plants are vital for health and growth promotion as well as preventing many diseases. [35]. The nutritional analysis of *B. indica* showed the presence of fibers in a high amount, followed by carbohydrates and proteins, just like other nutritional plants [26,36]. The high ash value depicts a good amount of minerals in plants [8]. Minerals are very important for living organisms to maintain their physiological and biochemical functions. Plants are considered a good source of minerals but may also contain toxic heavy metals that may lead to dangerous consequences to human health when ingested [26,37]. The *B. indica* extract contained a significant amount of important minerals such as calcium, iron, and magnesium. These results were in line with other reported studies [38]. Heavy metals like lead, copper, chromium, and cadmium quantified in the extract did not exceed the safety threshold prescribed by the WHO [39]. Forage, having a considerable amount of elements and nutrients, improve the growth, reproduction, and performance of animals [24,40]. It is reported that *B. indica* is used as forage, which increases the performance, growth, and milk yield of cattle [24,41].

Crude drugs may also be assessed qualitatively by their fluorescence pattern [42]. The fluorescence analysis of the extract showed different fluorescent colors when treated with different organic and inorganic solvents and observed under daylight and UV light. Different fluorescent colors indicated the presence of multiple compounds in the plant, as claimed in previously reported studies [43]. Phytochemical analysis of the crude extract showed the presence of important secondary metabolites such as phenolics, flavonoids, tannins, and saponins, which were further quantified. The results of the current study were similar to the reported phytochemical profile of other plants of the genus *Bassia* like *B. scoporia*, and *B. muricata*, etc. [10,17,44]. The presence of phenols and flavonoids makes *B. indica* a plant of therapeutic potential. It is a well-documented fact that polyphenols are involved in various human health benefits and strongly linked with antioxidant, antimicrobial, anti-inflammatory, cardioprotective, and anticancer activities [45,46]. In a previous study, phenolic compounds exhibited significant antimicrobial activity [47], so the antimicrobial effects of *B. indica* observed in the current study may be attributed to phenols. Tannins are also polyphenols, which precipitate the proteins and form complexes with enzymes, thereby inhibiting their activity. Tannins are known for their antimicrobial activities and are used in wound healing and anti-inflammatory activities [48]. Saponins are glycosides and have many therapeutic activities including diuretic, anti-inflammatory, antimicrobial, cardioprotective, and many other biological activities [49]. The functional groups observed in the FTIR analysis of *B. indica* further confirmed the phytochemical results, and this test can be used to control the quality and standardization of the plant material, as conducted in previously reported studies [50]. 1,2-Benzenedicarboxylic acid, diisooctyl ester, and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester were the most abundant compounds observed in the GC-MS analysis of the *n*-butanol fraction, and their presence has also been reported in many other medicinal plants with antimicrobial, anticancer, antifungal, antioxidant and anti-inflammatory properties [51–53]. Squalene observed in the current study has also been found in the phytochemical screening of many other plants, is considered as a natural antioxidant, and possesses cholesterol lowering, anticancer, immune enhancing, and chemopreventive activities [54]. Other identified compounds like 24,25 dihydroxycholecalciferol, oleic acid, 3-(octadecyloxy)propyl ester, azafrin, and rhodopin have also been identified in other plants and is reported to have calcium regulation, antifungal, cardioprotective, and antineoplastic activities, respectively [55–57]. In the ethyl acetate fraction, 4,4-dimethyladamantan-2-ol was the most abundant compound, and is also found in the essential oil of plants with antimalarial and antioxidant activities [58,59]. Other compounds like agathic acid, gitoxigenin, betulin, and stigmasterol have previously been isolated from other plants and shown to have antileishmanial and abortifacient [60], cardioprotective [61], antioxidant, anti-inflammatory, anticancer [62] anti-osteoarthritis, anti-inflammatory, and antibacterial [63] properties, respectively. Similarly, 5-methylfurfural, 2-methoxy-4-vinylphenol, Ar-turmerone, and hexadecanoic acid, methyl esters found in the *n*-hexane fraction have also been found in different plant extracts and reported to have acaricidal, anti-inflammatory, anticancer [64], anti-inflammatory, anti-nociceptive, anti-proliferative [65], and antibacterial activities [66], respectively. An oral acute toxicity study performed in mice and the MTT cytotoxicity assay carried out in mouse fibroblast cells showed that *B. indica* is safe and did not cause any toxicity.

The *B. indica* crude extract and all fractions showed good antibacterial effects against Gram-positive and Gram-negative strains. It was observed that the ethyl acetate fraction was more effective against bacterial strains, followed by the *n*-butanol fraction. The most abundant compound of the *n*-butanol fraction 1,2-benzenedicarboxylic acid, diisooctyl ester was previously isolated from the root of *Plumbago zeylanica* Linn and tested against different bacterial strains, which showed good antibacterial results [53,67]. Therefore, the present antibacterial results of the *n*-butanol fraction may be due to the presence of a large amount of the 1,2-benzenedicarboxylic acid, diisooctyl ester compound. Similarly, hexadecanoic acid, methyl ester, identified in the *n*-hexane fraction, was also isolated from clove, which showed antibacterial effects [66]. Furthermore, in the current study, it was observed that the

*B. indica* extract had a considerable amount of polyphenols and tannins, and the observed antibacterial effects may also be attributed to these secondary metabolites [48]. Moreover, to provide a theoretical rectification to the antibacterial investigations, the major compounds of different fractions identified in the GC-MS analysis were subjected to in silico computational analysis against the DNA gyrase subunit B of various bacteria. Computational studies like molecular docking help to predict the least good binding energy and high affinity as well as a better framework between protein and ligand interactions [68]. The amino acid residues interact with the DNA gyrase through hydrogen bonds, pi-cation, pi-sigma, pi-alkyl, and alkyl interactions, which play an important role in the overall stability and specificity of the binding between the two molecules [69].

#### 4. Materials and Methods

##### 4.1. Plant Collection

Aerial parts (Stem, branches and leaves) of *Bassia indica* (Wight) A.J. Scott were collected in bulk (10 kg) from the district pf Muzaffargarh (Punjab) in Pakistan [31°10'5" N 70°50'25" E] in June 2021, early morning before flowering, and were identified by a botanist, Dr. Ghulam Sarwar, Department of Botany, The Islamia University of Bahawalpur. The specimen was stored and voucher no. 324/Botany was issued. Plant material was cleaned, shade dried, and crushed into a coarse powder. A small amount of powder was stored in closed amber glass bottles for microscopic analysis.

##### 4.2. Extract Preparation and Fractionation

Plant extraction was performed as described previously with minor modifications [70]. Briefly, the powdered plant material (3 kg) was soaked into an aqueous methanol (30:70) solvent for 7 days with daily stirring and shaking. The soaked material was filtered and evaporated under reduced pressure and temperature until a thick semisolid crude extract was obtained. Then, 150 g of the extract was used for fractionation, and the remaining portion was stored in a freezer at  $-20\text{ }^{\circ}\text{C}$  for further use. The compounds present in the extract were then separated depending upon their polarity by the separation funnel method [71]. Solvents of different polarities were selected for this purpose, and 150 g of extract was dissolved in 250 mL of distilled water and extracted successively with an equal volume of different organic solvents (i.e., *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol, respectively). All of the solvent fractions were then evaporated under reduced pressure (150 mbr) and temperature ( $40\text{ }^{\circ}\text{C}$ ) to obtain thick extracts. The percentage yield of the crude extract and fractions was calculated, and the extracts were stored in closed containers at  $-20\text{ }^{\circ}\text{C}$  for further studies.

##### 4.3. Experimental Animals

Albino mice of either sex weighing about 30–50 g were housed in the animal house of the Pharmacology Research Lab, Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur. The animals were housed in polycarbonate cages with sawdust (renewed every 48 h) under standard lab conditions (temp:  $25 \pm 2\text{ }^{\circ}\text{C}$ ; humidity  $60 \pm 5\%$ ), a light and dark cycle of 12 h was maintained, and they were fed standard diet and water ad libitum. The study was carried out according to the guidelines of the Pharmacy Animal Ethics Committee (AEC file no PAEC/23/101).

##### 4.4. Pharmacognostic Evaluation

###### 4.4.1. Macroscopic Studies

Cleaned and shade dried samples of the *B. indica* plant were observed for color, shape, size, odor, taste, and texture. Similarly, the leaves and stem were powdered and observed for color, odor, and taste [72].

#### 4.4.2. Microscopic Studies

Microscopic studies of the *B. indica* fresh leaves and stem were performed under a simple compound microscope. Fresh, cleaned, small-sized (5–10 mm) parts of the leaf and stem were placed into the potato block and sections were cut with a razor blade manually and dipped into water in a Petri dish. Uniform and small-sized sections (5–15  $\mu\text{m}$ ) were stained and observed under a camera-fitted microscope (4 $\times$  and 10 $\times$ ) [72]. Similarly, dried powder of the aerial parts of *B. indica* was investigated following the previously described method with slight modifications [73]. A few particles of fine powder material were placed on cleaned glass slides and 1–2 drops of 10% chloral hydrate, 50% glycerin, and 5% iodine solution was added separately and mixed well with a needle. Glass cover slides were placed, and various structures were observed under the microscope at 10 $\times$ , 50 $\times$ , and 100 $\times$ .

#### 4.4.3. Scanning Electron Microscopy

The surface morphology of the powder was assessed by keeping the powder in the chamber of a scanning electron microscope. Scanning was performed at various magnifications ranging from 900 to 4500  $\text{cm}^{-1}$  at 10.0, 20.0, and 100.0  $\mu\text{m}$  [74].

#### 4.4.4. Swelling Index

The swelling index was calculated by adding 2 g of plant powder in a 100 mL measuring cylinder with 50 mL of water. The cylinder was gently shaken many times, allowed to stand for 24 h, then the volume occupied by the sample was measured [75].

#### 4.4.5. Extractive Value Determination

The plant powder (2 g) was macerated in *n*-hexane, ethyl acetate, dichloromethane, *n*-butanol, and distilled water (20 mL each) separately in air tight bottles for 7 days with intermittent shaking. The extract was filtered in pre-weighted flasks, then the filtrate was evaporated and the flasks were weighed again. The extractive value (percentage) was calculated using the following formula [72]:

$$\text{Extractive value(\%)} = \frac{\text{weight of extract}}{\text{weight of powder}} \times 100 \quad (1)$$

#### 4.4.6. Elemental and Nutritional Analysis

The quantities of the different metals and minerals in the extract were measured with the help of an atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Tokyo, Japan) under the conditions described in the AOAC (1990). Metals assessed in the extract were calcium (Ca), chromium (Cr), copper (Cu), cadmium (Cd), iron (Fe), lead (Pb), manganese (Mn), magnesium (Mg), nickel (Ni), and zinc (Zn). Commercially available stock solution (Applichem<sup>®</sup>, Darmstadt, Germany) was used for the preparation of calibrated standards in purified de-ionized water (1000 ppm). The glass apparatus used during the procedure of experimental work was kept overnight in 8 N  $\text{HNO}_3$  and cleaned with de-ionized water many times before use [76]. Nutritional contents such as carbohydrates, fats, proteins, and fiber percentage were calculated according to the AOAC 2005 guidelines and method adopted by Zaman et al. The carbohydrate percentage and total energy was calculated by the following equations [26]:

$$\text{Carbohydrate content} = 100 - (\% \text{moisture} + \% \text{protein} + \% \text{crude lipid} + \text{crude fiber} + \% \text{ash}) \quad (2)$$

$$\text{Total caloric value} \left( \frac{\text{Kcal}}{100 \text{ g}} \right) = (2.62 \times \text{protein}) + (4.2 \times \text{carbohydrate}) + 8.37 \times \text{fat} \quad (3)$$

#### 4.4.7. Fluorescence Analysis

A small quantity of the powder was soaked in different reagents as mentioned including methanol, ethyl acetate, dichloromethane, chloroform, *n*-butanol, *n*-hexane, 50%



HNO<sub>3</sub>, picric acid, conc. H<sub>2</sub>SO<sub>4</sub>, 50% HCl, 1% NaOH, iodine, and 5% FeCl<sub>3</sub>. After 5 min, all mixtures were observed in visible light and under a UV lamp at 254 nm for fluorescence [5].

#### 4.4.8. Thin Layer Chromatography

Thin layer chromatography of the plant extract and its fractions was carried out using various mobile phases of different concentrations and silica gel 60 backed aluminum TLC plates. The mobile phase was poured into a closed tank, and saturation was achieved by placing the filter paper across the walls of the tank. The sample extracts dissolved in different solvents were placed on a TLC plate with the help of a capillary tube, 1.5 cm above the base of the plate, and allowed to dry. Then, the plates were placed in a chromatographic tank until the elevation of the mobile phase to the upper mark. Then, the plates were removed, dried, and observed under a UV lamp ( $\lambda = 254$  nm) to visualize the separated compound spots, and their R<sub>f</sub> value was calculated [77].

### 4.5. Phytochemical Analysis

#### 4.5.1. Qualitative Analysis

For the detection of various phytochemicals present in the crude extract, a standard procedure was followed with some modifications [70]. Briefly, Molisch's test was used for the detection of carbohydrates, which turns a purple color in their presence. The formation of a yellow precipitate in Hager's reagent confirmed the alkaloids. The extract was treated with FeCl<sub>3</sub> and H<sub>2</sub>SO<sub>4</sub> for the detection of phenol and flavonoids, respectively. A brown colored ring at the interface in the Keller–Kiliani test showed the presence of glycosides in the extract. A blackish precipitate with the ferric chloride solution and a pink color with HCl confirmed the tannins and resins in the extract, respectively. The formation of persistent froth while vigorous shaking of the extract with water exhibited the presence of saponins.

#### 4.5.2. Quantitative Analysis of Total Phenols, Tannins, Flavonoids, and Saponins

The total phenol and tannins were estimated using the Folin–Ciocalteu method. A standard calibration curve was constructed using tannic acid solution. Different volumes (0, 20, 40, 60, 80, and 100  $\mu$ L) of standard tannic acid solution (10 mg/mL) were poured into a test tube and the final volume (500  $\mu$ L) was made with distilled water. Solutions were mixed with 250  $\mu$ L 1 N FC reagent and 1.25 mL of 20% sodium carbonate, and the absorbance was measured ( $\lambda = 725$  nm) after 40 min of incubation at room temperature. Similarly, 10, 30, and 100  $\mu$ L of the extract stock solution (100 mg/mL) were diluted and mixed with FC reagent and sodium carbonate, and the absorbance was measured after incubation. The total phenols were estimated as tannic acid equivalent from the calibration curve and the results were expressed as the total phenolic milligram per gram of extract on a dry matter basis. Total tannins were determined by separating and subtracting non-tannin phenols from the total phenols. For the estimation of non-tannin phenols, 1 mL of each extract stock solution (100 mg/mL) and distilled water was mixed with 100 mg polyvinyl pyrrolidone (PVPP), vortexed, and incubated at 4 °C for 15 min. Then, the mixture was centrifuged at 3000 rpm for 10 min, and the supernatant, containing non-tannin phenol, was collected. Next, 20, 60, and 200  $\mu$ L of the supernatant were diluted and mixed with FC reagent and sodium carbonate as above-mentioned, and the absorbance was taken. Values were interpolated against the tannic acid calibration curve [78]. The tannin contents were calculated with the following equation

$$\text{Tannins\%} = (\% \text{total phenolics}) - (\% \text{non-tannin phenolics}) \quad (4)$$

For the estimation of the total flavonoid content, 50  $\mu$ L of the extract sample (1 mg/mL) was mixed with 100  $\mu$ L methanol in a 96-well microplate. Then, 20  $\mu$ L 10% AlCl<sub>3</sub> was added in the well and gently shaken. After 3 min of incubation, 20  $\mu$ L 1 M CH<sub>3</sub>COONa was added to the well, followed by the addition of 60  $\mu$ L of methanol. One well containing 150  $\mu$ L methanol was used as the blank. The microplate was then further incubated for

40 min in the dark and the absorbance was measured at 430 nm using a microplate reader. A standard curve of quercetin (concentration ranging from 7.81 to 500 mg/mL, 2-fold dilution) was drawn under the same procedure. Flavonoid contents were expressed in milligram equivalent to quercetin per gram of dry extract (mg QE/g) [79].

The total saponin content present in the extract and its aqueous fraction were estimated by treating 1 g of extract with 20% acetic acid in ethanol and allowed to stand for 24 h at 50 °C. The mixture was filtered, concentrated in a water bath to one quarter of its initial volume, and then precipitated with NH<sub>4</sub>OH. Precipitates were separated by filtration, and the saponin content was calculated with the following equation [80].

$$\text{Saponin content} = \frac{W_2 - W_1}{\text{Sample weight}} \times 100 \quad (5)$$

where  $W_2$  is the weight of the filter paper + residue and  $W_1$  is the weight of the empty filter paper.

#### 4.6. FTIR Spectroscopy

Extracts can also be standardized by identifying different functional groups through FTIR analysis. The FTIR analysis of the extract was carried out at room temperature using a Bruker FTIR (Tensor 27 series, Ettlingen, Germany) scanning over the frequency range of 4000–650 cm<sup>−1</sup> at a resolution of 4 cm<sup>−1</sup>. Attenuated total reflectance (ATR) technology along with OPUS data collection software (version 7.8) was used to collect the spectra. A sample of the extract was kept on the pike miracle ATR cell with a zinc selenide crystal surface, followed by rotation of the assembly, thus forming a compact mass. Finally, the spectrum was scanned and recorded [81].

#### 4.7. GC-MS Analysis

GC-MS analysis of the *n*-butanol, ethyl acetate, and *n*-hexane fractions of the *B. indica* extract was performed by following the previously described method [82]. The samples were analyzed using a Thermo Scientific (DSQI) GC (Waltham, MA, USA) attached to the NIST11.L library. The gas was equipped with a TR-5MS capillary column with a length of 30 M, film thickness of 0.25 µm, and an internal diameter of 0.25 mm. Helium was used as the carrier gas with a 1 mL/min real flow rate. The GC-MS spectral lines were detected by the ionization energy method with an ionization energy of 70 electron volt and a 0.2 s scan time with a ranging fragment from 40 to 600 *m/z*. The injector was operated in split mode with a temperature of 250 °C. The 1 µL sample volume was injected with an initial oven temperature of 50 °C and held for 2 min, then increased to 150 °C with the temperature rate of 8 °C/min, and further increased to 300 °C with the temperature rate of 15 °C/min and held for 5 min. Retention time per minute, peak area, peak height, and spectral line patterns were used to identify the components present in the sample plant materials when compared with spectral lines from the database of authenticated compounds stored in the National Institute of Standards and Technology (NIST) library.

#### 4.8. Cytotoxicity Study

Cytotoxic effects were assessed by the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay on mouse fibroblast 3T3 cells. Cytotoxic effects were measured as concentration causing 50% cell growth inhibition (IC<sub>50</sub>) [83]. Percent inhibition was calculated by using the following formula:

$$\% \text{inhibition} = 100 - \frac{(\text{mean of O.D of test compound} - \text{mean of O.D of negative control})}{(\text{mean of O.D of positive control} - \text{mean of O.D of negative control})} \times 100 \quad (6)$$

#### 4.9. Acute Toxicity Study

The acute toxicity study was performed on mice with a weight of 28 ± 4 g according to OECD guidelines test no. 425 [84]. A limit test was used for the toxicity study. Animals

were housed under standard conditions and fed according to the mentioned guidelines. Initially, 2000 mg/kg of the extract was administered to a single mouse and the animal was closely monitored for the first 30 min, then for 4 h, and 24 h. After the survival of the first mouse, four additional mice were treated with the same dose and monitored accordingly. After that, the monitoring of all five mice was continued for 14 days, during which any signs of toxicity and altered behavior were observed.

#### 4.10. Antibacterial Activity

The antibacterial activity of the *B. indica* crude extract, its *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol, and aqueous fractions, and positive control (ceftriaxone) was assessed against Gram-positive bacteria (i.e., *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538)) and Gram-negative bacteria including *Escherichia coli* (ATCC 10536), *Salmonella typhi* (ATCC 19430), and *Pseudomonas aeruginosa* (ATCC 9027), as described previously with minor modifications [85]. Ceftriaxone was used as a standard due to its broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. All of the apparatus and solutions used were sterilized, and the experiment was carried out in triplicate. Antibacterial potential was measured by the following methods.

##### 4.10.1. Zone of Inhibition by Disc Diffusion Method

Solutions of different concentrations (10, 30, and 100 mg/mL) of the *B. indica* crude extract and its *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol, and aqueous fractions were prepared to assess the zone of inhibition. Discs of 5 mm were cut from filter paper and soaked in different concentrations of extracts or solvent (control) and dried at room temperature. Bacterial cultures of all bacterial strains were prepared in nutrient broth, incubated for 24 h at 37 °C, and diluted with a normal saline concentration equivalent to the 0.5 McFarland standard. Muller–Hinton agar was dissolved in distilled water, heated, and sterilized by autoclaving. Sterilized agar was poured into a Petri dish, marked into four equal parts, in a laminar airflow hood near a flame burner and was allowed to solidify. Bacterial culture (100 µL) of different strains was spread over agar, and discs soaked in different concentrations of the extract and positive control ceftriaxone (1 mg/mL) were placed. Petri dishes were incubated for 24 h at 37 °C and the zone of inhibition was measured.

##### 4.10.2. Minimum Inhibitory Concentration

The MIC of the extracts was measured using the 2-fold broth micro-dilution method in nutrient broth. Sterile 96-well plates were used for testing and dilutions ranging from 250 to 0.488 mg/mL were prepared by adding 100 µL of the extract concentration and 100 µL of nutrient broth. Negative control wells contained only 100 µL of nutrient broth. Stock solutions of the *B. indica* fractions were prepared in 10% DMSO. The effect of DMSO was ruled out by keeping one lane for the solvent without any extract. Similar dilutions of the standard drug ceftriaxone (dissolved in sterile distilled water) were prepared. Fresh bacterial inoculum, prepared from 24 h old culture, was standardized with 0.5 McFarland and 5 µL was added in each well. After 24 h of incubation at 37 °C, the clarity of the wells was observed to assess the MICs and the concentration of the wells was noted until no bacterial growth was seen [86].

#### 4.11. Molecular Docking

Molecular docking was performed by AutoDock Vina (version 4.2) software to analyze the binding interactions of different compounds, obtained from GC-MS data, within the active pockets of the DNA gyrase of the targeted bacteria. The study was carried out using the 3D crystal structure of the DNA gyrase subunit B of various bacteria, retrieved from the Protein Data Bank (www.rcsb.com) with PDB ID: 4DDQ for *B. subtilis*, 1KZN for *E. coli*, 7PTF for *P. aeruginosa*, 6FM4 for *S. aureus*, and 5ZTJ for *S. typhi* [87]. Before the docking analysis, the targeted DNA gyrase structure was prepared by using MGL tools,

where the heteroatoms and water molecules were removed, afterward, polar hydrogen and Kollman charges were added and the structure was rendered for missing amino acid residues [88]. The energy minimized 3D structures of all compounds were drawn using ChemDraw (Ultra 12.0) 3D [89]. The docking protocol was validated by first separating the co-crystal ligand from the active pocket of the complex, and then re-docking was performed to validate its accuracy [90]. The force field MMFF94x with an RMSD gradient of  $>0.01 \text{ kcal} \cdot \text{mol}^{-1} \text{ \AA}^{-1}$  was used to minimize the energy of the selected compounds [91]. All compounds were docked within the active pocket of the DNA gyrase subunit B using AutoDock's default genetic algorithm as the scoring function. The dimensions of the grid box were set as (x;  $-41.857501$  y;  $-12.318299$  z;  $-8.245484$  for 4DDQ, x;  $19.537493$ , y;  $19.165566$ , z;  $43.283299$  for 1KZN, x;  $-2.468462$  y;  $-2.933290$  z;  $38.392610$  for 7PTF, x;  $-42.133240$  y;  $-3.919944$  z;  $22.992590$  for 6FM4 and x;  $26.379112$ , y;  $22.904972$ , z;  $22.022154$  for 5ZTJ). About 100 different configurations of the docking complexes were generated for the compounds with the active pocket of DNA gyrase subunit B. Finally, the most stable configuration possessing high affinity was selected for the further analysis and development of 2D and 3D models to understand the binding interactions of the compound within the active site of proteins [92]. For a more comprehensive understanding of the binding affinity, we also calculated and presented the binding energies of various other compounds. These binding energies can provide insights into the strength and stability of the binding interactions between these compounds and the DNA gyrase. Moreover, the results were compared with the docking of the positive control and the procedure was validated via the re-docking procedure.

#### 4.12. Statistical Analysis

The results were calculated by linear regression using GraphPad Prism 6 and the results were presented as the mean  $\pm$  SEM.

## 5. Conclusions

Our work has set forth pharmacognostic standards for the correct identification and authentication required for the quality control of *Bassia indica* (Wight) A.J. Scott. The results showed that the plant is rich in minerals and nutrients. Chemical characterization of the extract showed the presence of important bioactive phytochemicals like carbohydrates, glycosides, phenols, saponins, and sterols. GC-MS analysis identified several phytochemicals of pharmacological importance. *B. indica* showed good antibacterial activity against both Gram-positive and Gram-negative bacteria. Molecular docking studies described several phytochemicals with good binding interaction with DNA gyrase, which may suggest its antibacterial mechanism.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants13131753/s1>, Figure S1. The predicted 3D(A) and 2D(B) binding mode of the co-crystal ligand against the DNA gyrase subunit B of *B. subtilis*; Figure S2. The predicted 3D(A) and 2D(B) binding mode of ceftriaxone against the DNA gyrase subunit B of *B. subtilis*; Figure S3. The predicted 3D(A) and 2D(B) binding mode of the co-crystal ligand against the DNA gyrase subunit B of *E. coli*; Figure S4. The predicted 3D(A) and 2D(B) binding mode of ceftriaxone against the DNA gyrase subunit B of *E. coli*; Figure S5. The predicted 3D(A) and 2D(B) binding mode of the co-crystal ligand against the DNA gyrase subunit B of *P. aeruginosa*; Figure S6. The predicted 3D(A) and 2D(B) binding mode of ceftriaxone against the DNA gyrase subunit B of *P. aeruginosa*; Figure S7. The predicted 3D(A) and 2D(B) binding mode of the co-crystal ligand against the DNA gyrase subunit B of *S. aureus*; Figure S8. The predicted 3D(A) and 2D(B) binding mode of ceftriaxone against the DNA gyrase subunit B of *S. aureus*; Figure S9. The predicted 3D(A) and 2D(B) binding mode of ceftriaxone against the DNA gyrase subunit B of *S. typhi*; Table S1. Molecular docking scores of the co-crystal ligands with the targeted proteins.

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## Article

# Phytochemical Composition and Antibacterial Activity of *Barleria allostellata* C.B. Clarke Leaf and Stem Extracts

Serisha Gangaram <sup>1,\*</sup>, Yougasphree Naidoo <sup>1</sup>, Yaser Hassan Dewir <sup>2</sup>, Moganavelli Singh <sup>1</sup>, Johnson Lin <sup>1</sup> and Hosakatte Niranjana Murthy <sup>3</sup>

<sup>1</sup> School of Life Sciences, Westville Campus, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa

<sup>2</sup> Plant Production Department, College of Food and Agriculture Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia

<sup>3</sup> Department of Horticultural Science, Chungbuk National University, Cheongju 28644, Republic of Korea

\* Correspondence: serishagangaram@yahoo.com

**Abstract:** *Barleria allostellata* (Acanthaceae) is a shrub located in South Africa and is relatively understudied. However, plants within this genus are well known for their medicinal and ethnopharmacological properties. This study aimed to characterise the phytochemical compounds and antibacterial efficacies of *B. allostellata*. Phytochemical analysis, fluorescence microscopy and gas chromatography-mass spectrometry (GC-MS) analysis were performed to determine the composition of compounds that may be of medicinal importance. Crude leaf and stem extracts (hexane, chloroform and methanol) were subjected to an antibacterial analysis against several pathogenic microorganisms. The qualitative phytochemical screening of leaf and stem extracts revealed the presence various compounds. Fluorescence microscopy qualitatively assessed the leaf and stem powdered material, which displayed various colours under bright and UV light. GC-MS chromatograms represents 10–108 peaks of various compounds detected in the leaf and stem crude extracts. Major pharmacologically active compounds found in the extracts were alpha-amyrin, flavone, phenol, phytol, phytol acetate, squalene and stigmasterol. Crude extracts positively inhibited Gram-positive and Gram-negative bacteria. Significance was established at  $p < 0.05$  for all concentrations and treatments. These results indicate that the leaves and stems of *B. allostellata* are rich in bioactive compounds, which could be a potential source of antibacterial agents for treating various diseases linked to the pathogenic bacteria studied. Future discoveries from this plant could advance the use of indigenous traditional medicine and provide novel drug leads.

**Keywords:** antibacterial activity; gas chromatography-mass spectrometry; phytochemical analysis

## 1. Introduction

The dependence on plants as a source of medicine is prevalent in developing countries, especially where traditional medicine plays an important role in health care [1–3]. As defined by the World Health Organisation (WHO), traditional medicine is the knowledge, skill and practices based on the beliefs and experiences in various cultures [4]. The demand for herbal medicines worldwide is rapidly increasing due to their availability, low cost and higher safety margins [5–7]. The adverse side-effects of conventional medicine are related to certain pharmacological compounds; therefore, creating different therapies with greater effectiveness and bioavailability, with fewer side-effects, is essential [8,9]. Natural compounds isolated from plants have been assumed to remain an essential part of the exploration for new medicines against human diseases [10,11].

Africa is considered the cradle of humankind, comprising a rich cultural and biological diversity and with healing practices varying in regions [5,7,12]. Infectious diseases are a serious problem in Africa [13,14], with one of the leading causes of morbidity and mortality arising from bacterial infections (Gram-positive and Gram-negative bacteria) [15–18]. However, with



the use of herbal medicine, certain bacterial infections have been reported to be under control, while others are resistant [19]. According to the WHO, by 2050, there will be approximately 10 million deaths arising from common diseases such as respiratory and urinary tract infections and drug-resistant pathogens, surpassing the number of deaths resulting from cancer [20,21]. Therefore, it is crucial to find alternative solutions, such as herbal extracts, to overcome future threats in the medical field [22].

Throughout the history of mankind, plant extracts have been used to treat various ailments through herbal preparations [23–26]. These preparations include concoctions, decoctions, infusions and teas [27,28]. Plants are rich in several naturally occurring phytochemicals such as alkaloids, flavonoids, tannins and terpenoids, which have been found to possess antimicrobial properties. These secondary metabolites are important components of a plant's natural defence mechanisms and are products of primary metabolism [29–36].

In South Africa, about 3000 medicinal plants were reported to be used regularly, and from these plants, 38 indigenous species were commercialised [37,38]. These commercialised plant species are available as processed material in various forms, such as capsules, ointments, tablets or teas [39,40]. The verified record of natural products being used in drug discovery [41,42] has provided compelling evidence for increasing the exploration of nature to identify unique active compounds as promising leads for effective drug development [43–45]. There has been significant progress in pharmaceutical industries in search of important plant-based medicinal compounds; however, a significant amount of the plant biodiversity remains unexplored [46,47]. Screening plants for biologically active compounds has resulted in the development of new therapeutic drugs to treat several diseases [48].

*Barleria allostellata* C.B. Clarke (Acanthaceae) is a shrub located in South Africa. This shrub extends from Limpopo, Gauteng and Mpumalanga to KwaZulu-Natal [49]. Although *B. allostellata* has no recorded practice in traditional medicine, many species within the genus have been widely used in traditional medicine and were confirmed to contain various compounds possessing biological effects such as analgesic, anti-inflammatory, antileukaemic, antihyperglycemic, antitumour, anti-amoebic, antibiotic and virucidal activities [50–55]. Amoo et al. [56] examined the antioxidant potential of the methanolic extracts of the leaves and stems of *B. allostellata* and found the greatest flavonoid content in the stems of *B. allostellata* stems. Additionally, Amoo et al. [57] verified the antibacterial, antifungal and anti-inflammatory activity of *B. allostellata* against *B. subtilis*, *S. aureus* and *E. coli*. Thus, further investigation on *B. allostellata* is important as this study will provide baseline information on the potential usage of extracts from this plant. There is a scarcity of scientific data on the phytochemical compounds of the leaf and stem extracts of *B. allostellata* and its potential antibacterial activity against human pathogens. This study was therefore carried out to bridge these gaps.

## 2. Results and Discussion

### 2.1. Organoleptic Characteristics and Crude Extract Yield of *B. allostellata*

Organoleptic evaluation is a conventional, qualitative method whereby an individual uses their sight, smell, taste and touch to document the characteristic features of crude drugs. These assessments may serve as a baseline for preliminary phytochemical and pharmacological screening of a plant [58]. The organoleptic features of *B. allostellata* were evaluated by using sensory organs (Table 1). The following features were noted on both adaxial and abaxial surfaces: The leaves were grey-green in colour but lighter on the lower surface. The stems appeared as 'yellow-buff' on the upmost internodes and white/cream below. The odour of the leaves was slightly aromatic, whilst the stems were inodorous. The taste was acrid for both leaves and stems. Both surfaces of the leaves were velvety, whereas the stems were woody and glabrescent. According to Shaheen et al. [59], organoleptic studies are important taxonomic parameters, assisting in the verification of several medicinal plant species.



**Table 1.** Organoleptic features of different parts of *B. albostellata*.

| Organoleptic Features | Leaf                                                      | Stem                                                      |
|-----------------------|-----------------------------------------------------------|-----------------------------------------------------------|
| Colour                | Grey-green on both surfaces but lighter on the lower side | Yellow-buff on uppermost internodes, white or cream below |
| Odour                 | Slightly aromatic                                         | Inodorous                                                 |
| Taste                 | Acrid                                                     | Acrid                                                     |
| Texture               | Velvety                                                   | Woody, glabrescent                                        |

The highest percentage yield of the crude extracts from *B. albostellata* was obtained from the methanolic extract of the leaves (16.78%), followed by 9.38% from the methanolic stem extract (Table 2). The lowest percentage yield was observed in the hexane stem extract (1.94%). Overall, this suggests that the percentage yield of phyto-compounds in *B. albostellata* was greater in the leaf crude extract than from the stem. Furthermore, this implies that there may be more polar compounds in the leaf extracts and a smaller amount of non-polar compounds in the stem. Therefore, the yield obtained indicates the polarity of the different solvents is related to the plants' pharmacological importance [60,61]. Each crude extract (hexane, chloroform and methanol) displayed distinct colours (Table 2). Hexane extracts were oily upon evaporation of the solvent, whereas chloroform and methanol dried to a hard-sticky solid.

**Table 2.** Percentage yield of the leaf and stem crude extracts of *B. albostellata*.

| Crude Extract | Leaves                  | Stem  | Leaves               | Stem | Leaves      | Stem         |
|---------------|-------------------------|-------|----------------------|------|-------------|--------------|
|               | Dried Extract Yield (g) |       | Percentage Yield (%) |      | Colour      |              |
| Hexane        | 0.139                   | 0.194 | 1.39                 | 1.94 | Dark yellow | Light yellow |
| Chloroform    | 0.265                   | 0.219 | 2.65                 | 2.19 | Dark green  | Light green  |
| Methanol      | 1.678                   | 0.938 | 16.78                | 9.38 | Dark brown  | Light yellow |

## 2.2. Phytochemical Screening for Major Classes of Compounds in Extracts of *B. albostellata* Using Qualitative Colour Tests, TLC and Fluorescence Analysis

Major compounds identified in leaf and stem extracts of *B. albostellata* were alkaloids, amino acids, carbohydrates, flavonoids, mucilage and gums, phenols, saponins, terpenoids and sterols (Table 3). Fixed oils and fats were present in leaves and absent in the stem extracts. The intensity of compounds in the leaf extracts was greater in comparison to the stem. These compounds can act as defence mechanisms against various microorganisms, herbivores and insects [62–64].

Various phytochemicals have been known to contain diverse activities that may help protect against chronic diseases [65,66]. Amoo et al. [56] reported the presence of phenols, iridoids, gallotannins, flavonoids and condensed tannin in the leaves and stems of *B. albostellata*. These authors also found the total iridoid content to be the highest in the leaves of *B. albostellata*. Important pharmacological alkaloids can be found in iridoids, as this compound is known to be a precursor in the biosynthesis of alkaloids [67,68]. Similar compounds were also found in extracts of other *Barleria* species including *B. acuminata* [69], *B. dintteri* [10], *B. cristata* [70–72], *B. longiflora* [73] and *B. prionitis* [74].

Metabolites such as alkaloids and terpenoids (Table 3) were reported to contain antimicrobial, anticancer and anti-malarial properties [75–80]. Additionally, alkaloids have organic and natural constituents with sedative and analgesic roles [81]. Amino acids, carbohydrates and fixed oils and fats (Table 3) were reported to contain antioxidant properties [82–84]. Flavonoids and phenols (Table 3) possessed anti-inflammatory, anti-apoptosis, anti-carcinogen and anti-ageing properties [85–88].

**Table 3.** Preliminary phytochemical screening for major classes of compounds in hexane, chloroform and methanolic leaf and stem extracts of *B. albostellata*.

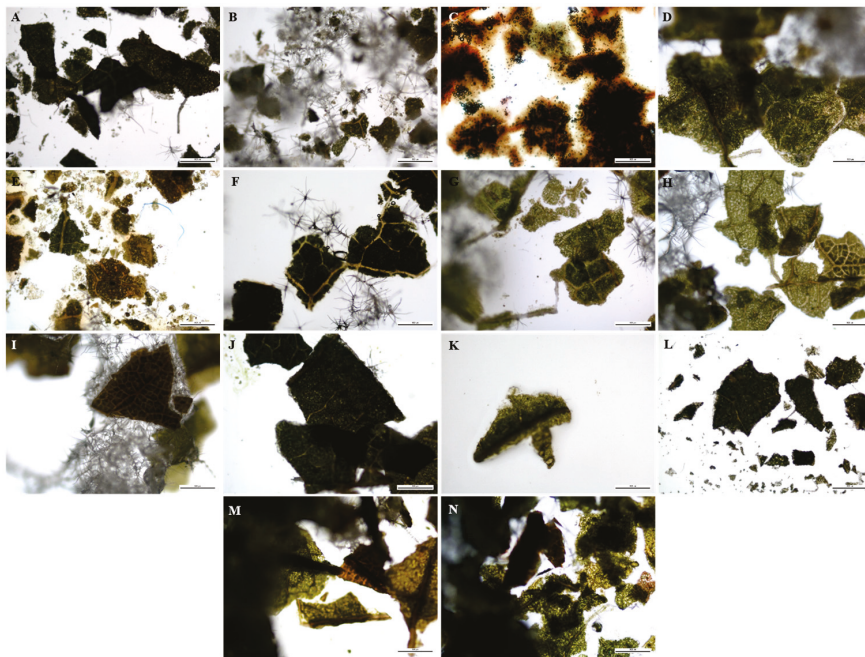
| Compound Group      | Phytochemical Test | Leaves |            |          | Stems  |            |          |
|---------------------|--------------------|--------|------------|----------|--------|------------|----------|
|                     |                    | Hexane | Chloroform | Methanol | Hexane | Chloroform | Methanol |
| Alkaloids           | Dragendorffs       | +++    | +++        | +++      | ++     | +++        | +++      |
|                     | Mayers             | +++    | —          | ++       | —      | —          | +-       |
|                     | Wagners            | +++    | +++        | +-       | +-     | +++        | +-       |
| Amino acids         | Ninhydrin          | +-     | —          | +-       | —      | —          | ++       |
| Carbohydrates       | Benedicts          | —      | —          | +++      | —      | ++         | —        |
|                     | Fehlings           | —      | +++        | +-       | +++    | +++        | ++       |
|                     | Molisch            | —      | +-         | +-       | —      | +-         | —        |
| Fixed oils and fats | Filter paper       | ++     | +-         | +-       | —      | —          | —        |
| Flavonoids          | Lead acetate       | +++    | +++        | +-       | +++    | +++        | +-       |
| Mucilage and Gums   | Ruthenium          | +++    | +++        | +++      | ++     | +++        | +++      |
|                     | Ferric trichloride | +-     | ++         | +++      | +-     | +-         | +-       |
| Saponins            | Froth              | +-     | ++         | +-       | +-     | +-         | ++       |
|                     | Foam               | +-     | +-         | +++      | +++    | +++        | ++       |
| Terpenoids          | Chloroform         | ++     | ++         | ++       | +-     | +-         | ++       |
| Sterols             | Salkowski's        | —      | ++         | +-       | —      | —          | +-       |

Intensity of reaction: (—) no observed changes, (+—) low intensity, (++) medium intensity, (+++) high intensity.

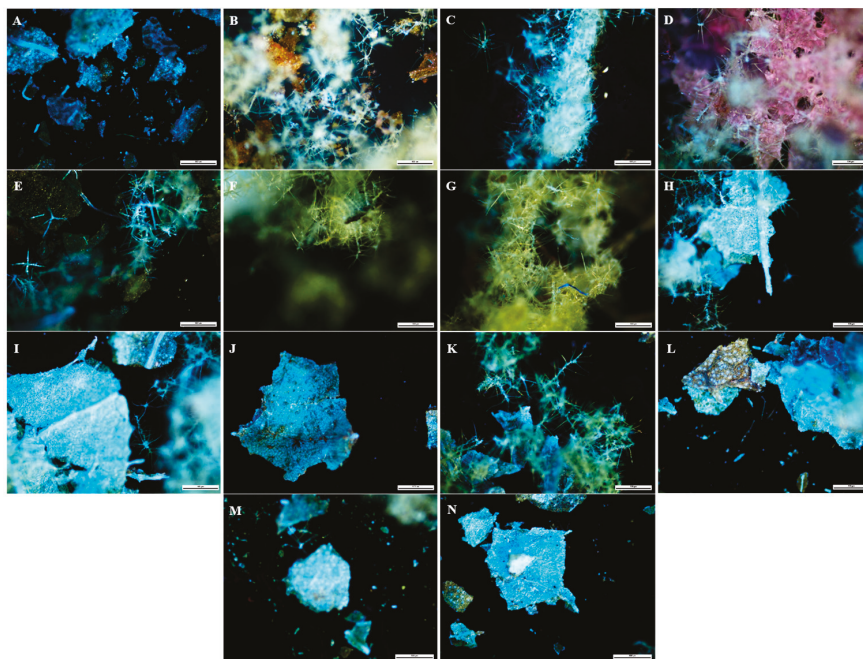
Mucilage and gums (Table 3) are used in the treatment of gastric ulcers, for wound healing and as cytoprotective agents, and also contain antipyretic and antiseptic properties [89–92]. Saponins present in plant extracts (Table 3) are believed to contain anticancer, antioxidant, antiviral and anti-inflammatory properties [93,94]. Additionally, saponins display several hepatoprotective and antimicrobial activities [95]. Sterols were found to reduce cholesterol levels and contain anti-inflammatory and antioxidant properties (Table 3) [96–100].

A technique used in the qualitative assessment of natural products or crude drugs is fluorescence analysis, which is an important tool for pharmacognostic evaluation [101,102]. The powdered and fluorescence characteristics of the leaf and stem powder of *B. albostellata* are presented in Figures 1–4. The powdered leaf and stem material treated with several reagents displayed various colours when observed under bright light, and this is compared to the colours observed under UV light. It should be noted that the colours indicated for the powdered leaf and stem material viewed under bright light were described according to the overall appearance. The purity and quality of crude drugs are occasionally authenticated using standard fluorescence characteristics, as certain natural products display no fluorescence in daylight but do so under UV light [103]. Natural products such as berberine alkaloids exhibit fluorescence under UV light but not in daylight [104,105]. As most crude drug materials do not fluoresce, these materials are converted either into fluorescent decomposition- or by-products with the aid of several reagents [101,102,106]. Furthermore, fluorescence analysis can be used to preserve the quality and effectiveness of crude drug materials by easily detecting adulterants and substituents [101,102,107,108].

The most prominent UV colour that stands out in both leaf and stem powder was blue (Figures 2 and 4). This colour was observed in multiple plant samples where different reagents were used. According to Chase and Pratt [109], several drugs display duplication of colours, as there are sometimes more than four drugs found in a particular colour group. According to Sridharan and Gounder [110], powdered leaves of *B. montana* were separately exposed to 24 h of light with the addition of various reagents. These included powder + water, + ethanol, + ethyl acetate, + hexane, + chloroform and + acetone. Colours observed from the various reagents after 24 h were orange, green, pale and light green, respectively. When these samples were exposed to UV light, colours observed were greenish-orange, light and dark green, pale yellow, yellowish-orange and pale red. Similar fluorescence results listed in this study were detected for certain reagents in the powdered leaf material for *B. noctiflora* [111] and *B. gibsoni* [112].

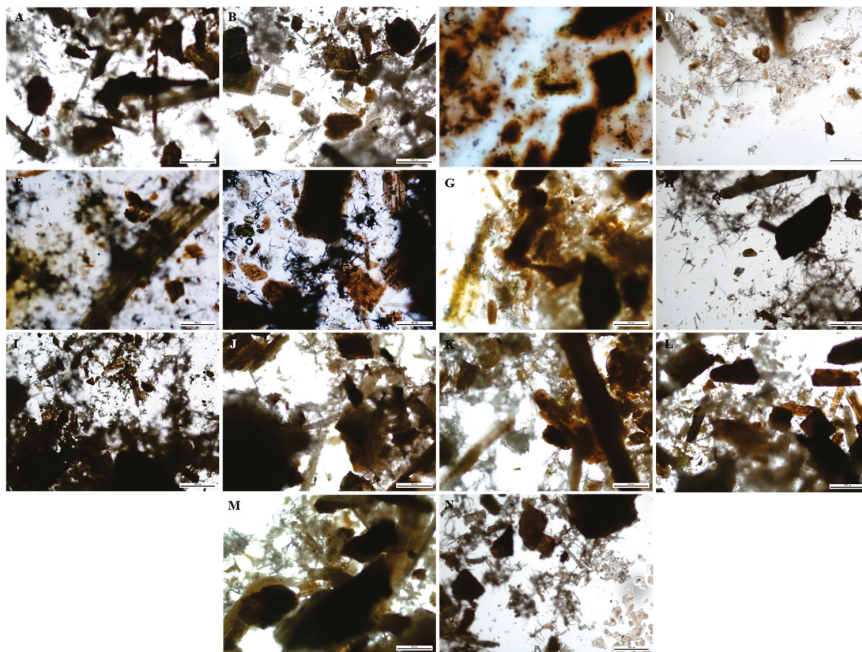


**Figure 1.** Leaf powder analysis (bright light) of *B. albostellata*. (A) Powder; (B) powder + water; (C) powder +  $H_2SO_4$ ; (D) powder + acetic acid; (E) powder + aqueous NaOH; (F) powder + HCl; (G) powder + ethanol; (H) powder + ethyl acetate; (I) powder + hexane; (J) powder + chloroform; (K) powder + methanol; (L) powder + petroleum ether; (M) powder + diethyl ether; (N) powder + acetone.

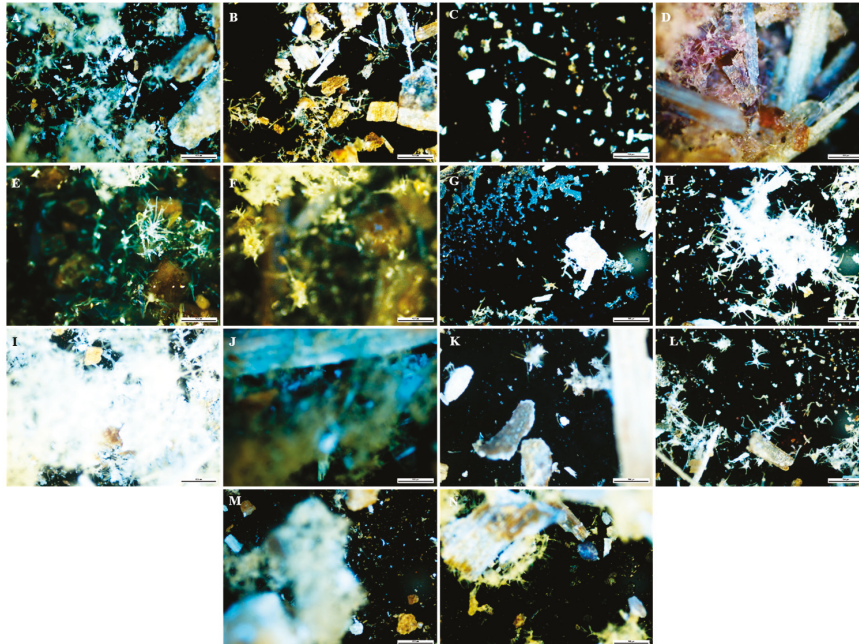


**Figure 2.** Fluorescence analysis (UV light) of leaf powder from *B. albostellata*. (A) Powder; (B) powder + water; (C) powder +  $H_2SO_4$ ; (D) powder + acetic acid; (E) powder + aqueous NaOH; (F) powder + HCl; (G) powder + ethanol; (H) powder + ethyl acetate; (I) powder + hexane; (J) powder + chloroform; (K) powder + methanol; (L) powder + petroleum ether; (M) powder + diethyl ether; (N) powder + acetone.





**Figure 3.** Stem powder analysis (bright light) from *B. albostellata*. (A) Powder; (B) powder + water; (C) powder +  $H_2SO_4$ ; (D) powder + acetic acid; (E) powder + aqueous NaOH; (F) powder + HCl; (G) powder + ethanol; (H) powder + ethyl acetate; (I) powder + hexane; (J) powder + chloroform; (K) powder + methanol; (L) powder + petroleum ether; (M) powder + diethyl ether; (N) powder + acetone.



**Figure 4.** Fluorescence analysis (UV light) of stem powder from *B. albostellata*. (A) Powder; (B) powder + water; (C) powder +  $H_2SO_4$ ; (D) powder + acetic acid; (E) powder + aqueous NaOH; (F) powder + HCl; (G) powder + ethanol; (H) powder + ethyl acetate; (I) powder + hexane; (J) powder + chloroform; (K) powder + methanol; (L) powder + petroleum ether; (M) powder + diethyl ether; (N) powder + acetone.

### 2.3. Phytochemical Screening for Major Classes of Compounds in *B. albostellata* Extracts Using GC-MS

The GC-MS chromatogram represents 10 peaks (Figure 5), 10 peaks (Figure 6) and 108 peaks (Figure 7) of various compounds detected in the leaf hexane, chloroform and methanolic extract, respectively. Additionally, 10 peaks (Figure 8), 10 peaks (Figure 9) and 104 peaks (Figure 10) were identified in the stem hexane, chloroform and methanolic extract. This analysis is used for the qualitative and quantitative examination of active compounds in plants [113,114]. Several overlapping peaks were observed in the middle stages of most chromatograms, Figure 5 (19–30 min), Figure 7 (14–33 min), Figure 8 (19–20 min), Figure 9 (19–25 min) and Figure 10 (19–33 min). Each peak in the chromatogram (Figures 5–10) represents a signal produced when a compound is washed out with a solvent from the gas chromatography column into the detector [115,116].

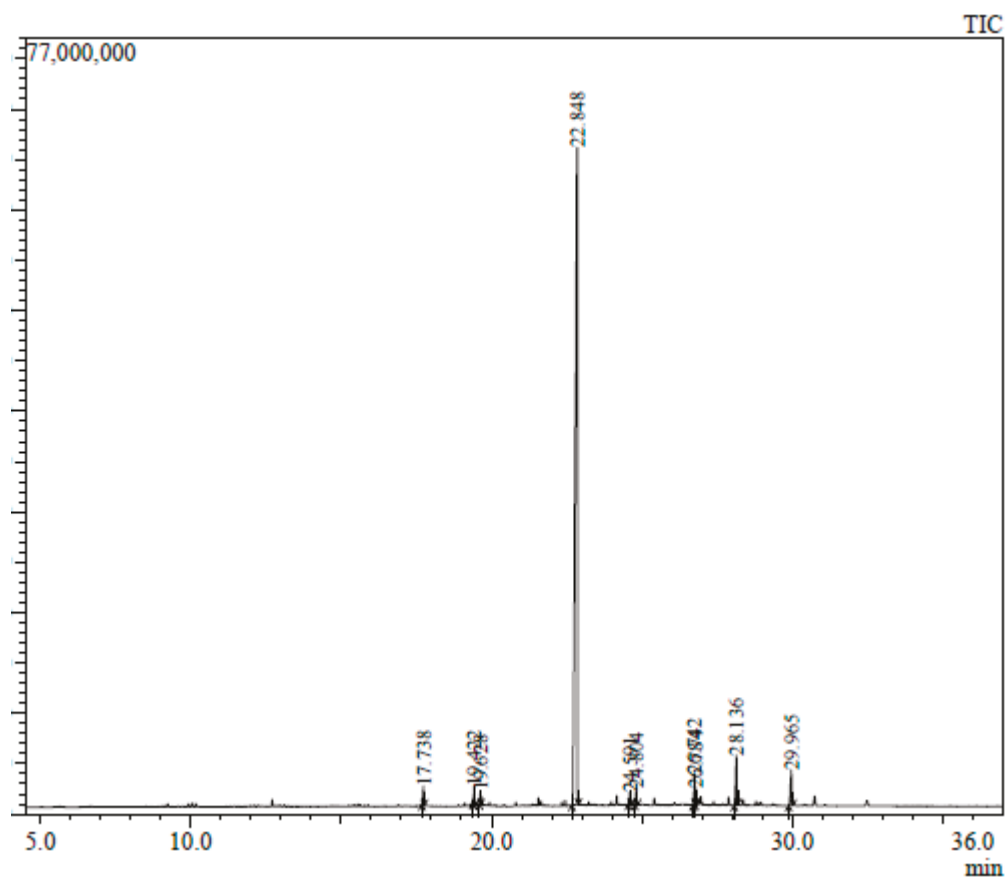


Figure 5. GC-MS chromatogram of leaf hexane extract of *B. albostellata*.

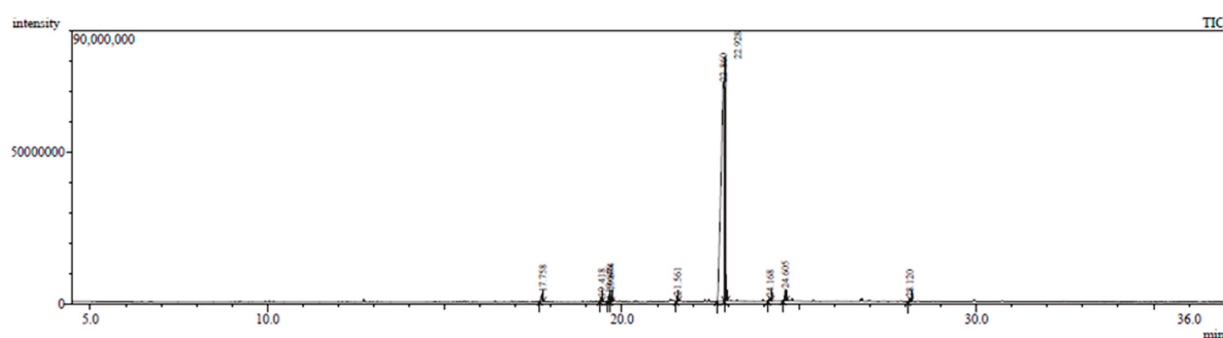
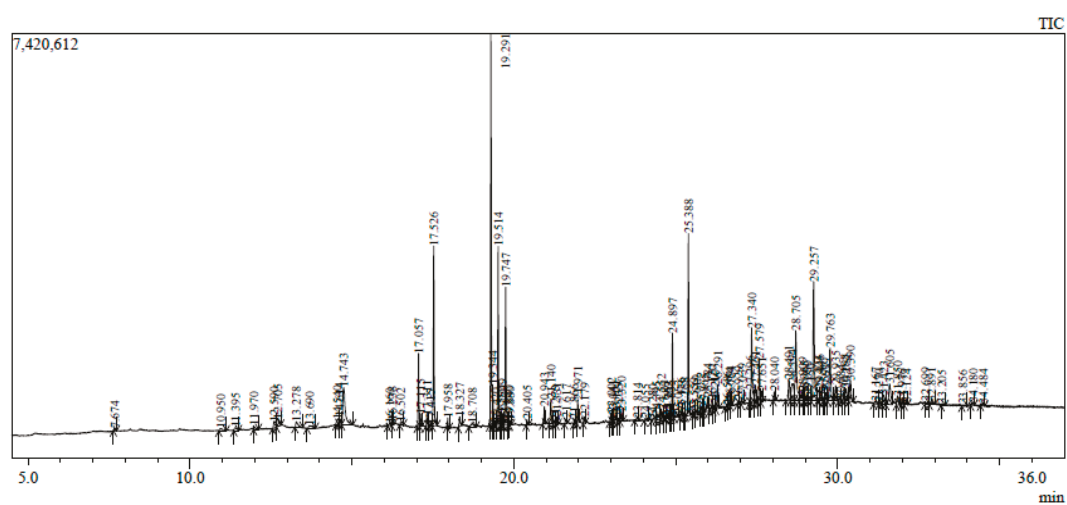
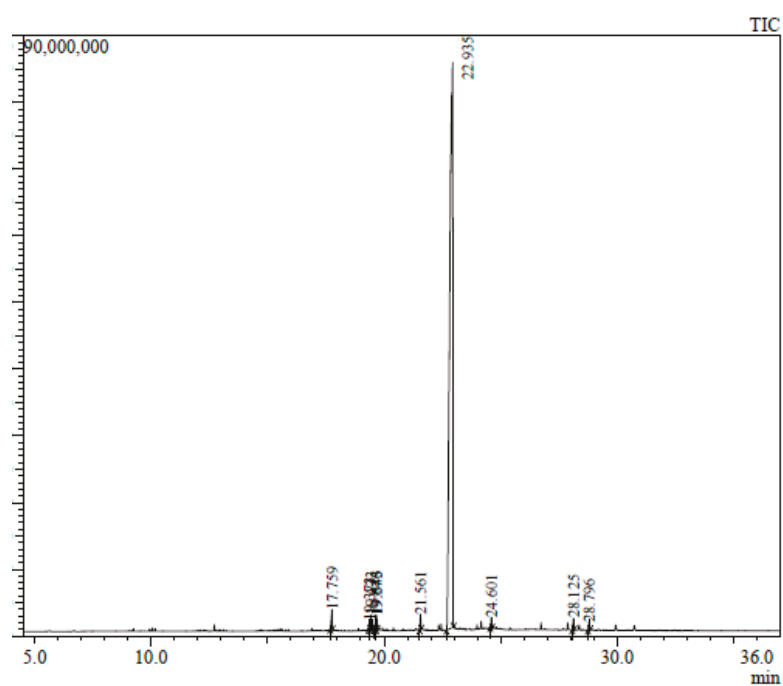


Figure 6. GC-MS chromatogram of leaf chloroform extract of *B. albostellata*.





**Figure 7.** GC-MS chromatogram of leaf methanol extract of *B. albostellata*.



**Figure 8.** GC-MS chromatogram of stem hexane extract of *B. albostellata*.

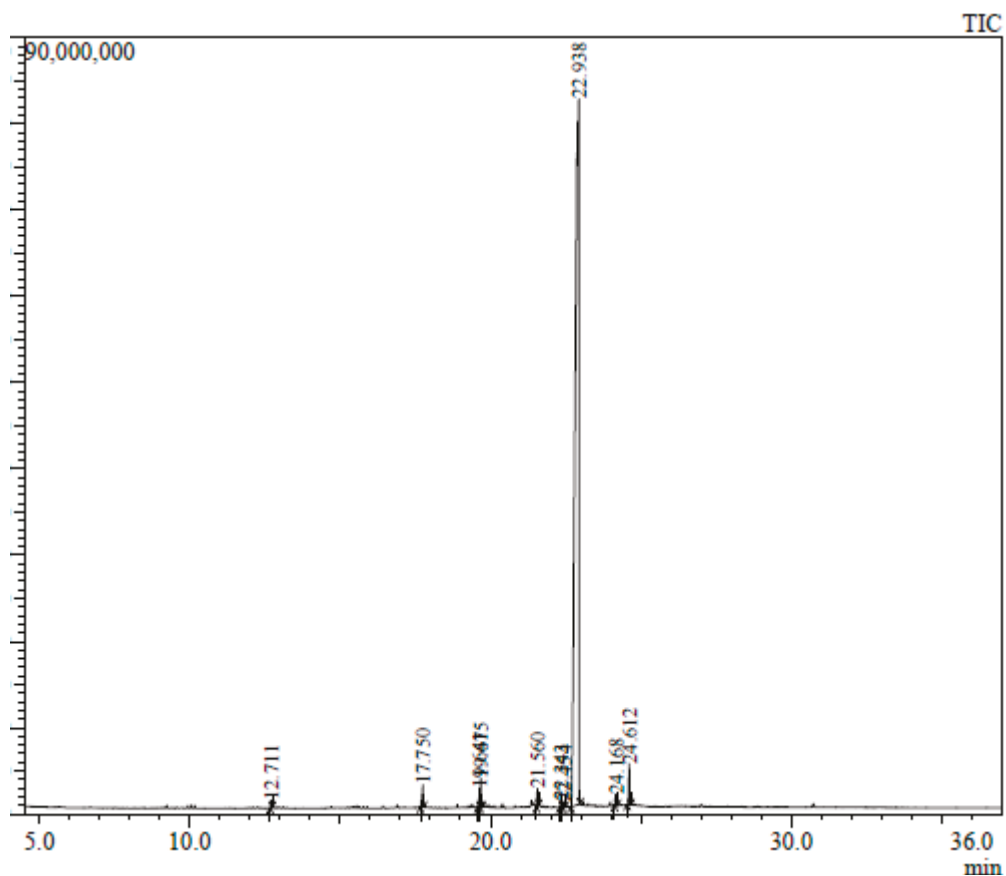


Figure 9. GC-MS chromatogram of stem chloroform extract of *B. allostellata*.

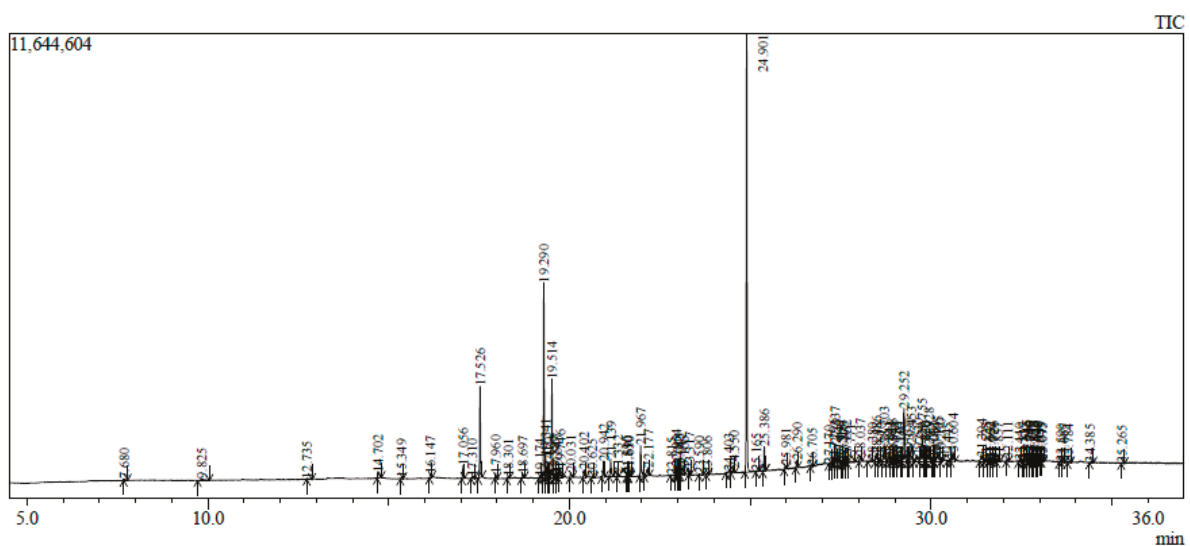


Figure 10. GC-MS chromatogram of stem methanol extract of *B. allostellata*.

Numerous small peaks were observed throughout the chromatogram. Major chemical compounds with high peaks and area percentages greater than one were selected and identified in leaf and stem extracts (hexane, chloroform and methanol) (Tables 4–9). In certain circumstances, compounds with an area percentage less than one were only mentioned if they played an important role in the genus. The methanolic extracts for both leaf and stem revealed the highest number of compounds. Compounds with an area percentage greater than one were found in the leaf (16) and stem (15) methanolic extracts (Tables 6 and 9).

**Table 4.** Phytochemical compounds identified in leaf hexane extracts of *B. allostellata* by GC-MS analysis.

| Peak | Retention Time | Phytochemical Compound                     | Molecular Formula                              | Molecular Weight | CAS No.    | Area % |
|------|----------------|--------------------------------------------|------------------------------------------------|------------------|------------|--------|
| 1    | 17.738         | Pentadecanoic acid                         | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub> | 242              | 1002-84-2  | 1.02   |
| 2    | 19.422         | 9,12,15-Octadecatrienoic acid, (Z)         | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub> | 278              | 463-40-1   | 1.25   |
| 3    | 19.628         | Octadecanoic acid                          | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> | 284              | 57-11-4    | 1.09   |
| 4    | 24.591         | 13-Docosenamide, (Z)                       | C <sub>22</sub> H <sub>43</sub> NO             | 337              | 112-84-5   | 1.12   |
| 5    | 24.804         | Squalene                                   | C <sub>30</sub> H <sub>50</sub>                | 410              | 111-02-4   | 1.06   |
| 6    | 26.742         | Eicosane                                   | C <sub>20</sub> H <sub>42</sub>                | 282              | 112-95-8   | 1.39   |
| 7    | 26.784         | 1-Heptacosanol                             | C <sub>27</sub> H <sub>56</sub> O              | 396              | 2004-39-9  | 1.21   |
| 8    | 28.136         | Tetratetracontane                          | C <sub>44</sub> H <sub>90</sub>                | 618              | 7098-22-8  | 3.25   |
| 9    | 29.965         | l-(+)-Ascorbic acid<br>2,6-dihexadecanoate | C <sub>38</sub> H <sub>68</sub> O <sub>8</sub> | 652              | 28474-90-0 | 2.85   |

**Table 5.** Phytochemical compounds identified in leaf chloroform extracts of *B. allostellata* by GC-MS analysis.

| Peak | Retention Time | Phytochemical Compound                          | Molecular Formula                              | Molecular Weight | CAS No.   | Area % |
|------|----------------|-------------------------------------------------|------------------------------------------------|------------------|-----------|--------|
| 1    | 17.738         | Pentadecanoic acid                              | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub> | 242              | 1002-84-2 | 1.02   |
| 2    | 19.418         | 9,12,15-Octadecatrienoic acid, (Z)              | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub> | 278              | 463-40-1  | 1.04   |
| 3.   | 19.646         | Tridecanoic acid                                | C <sub>13</sub> H <sub>26</sub> O <sub>2</sub> | 214              | 638-53-9  | 1.01   |
| 4    | 19.674         | Decanedioic acid, dibutyl ester                 | C <sub>18</sub> H <sub>34</sub> O              | 314              | 109-43-3  | 1.02   |
| 5    | 24.168         | Octadecanoic acid,<br>2,3-dihydroxypropyl ester | C <sub>21</sub> H <sub>42</sub> O              | 443              | 123-94-4  | 1.00   |
| 6    | 24.605         | 13-Docosenamide, (Z)                            | C <sub>22</sub> H <sub>43</sub> NO             | 338              | 112-84-5  | 1.03   |
| 7    | 28.120         | Tetratetracontane                               | C <sub>44</sub> H <sub>90</sub>                | 619              | 7098-22-8 | 1.00   |

**Table 6.** Phytochemical compounds identified in leaf methanol extracts of *B. allostellata* by GC-MS analysis.

| Peak | Retention Time | Phytochemical Compound                                   | Molecular Formula                              | Molecular Weight (g/mol) | CAS No.    | Area % |
|------|----------------|----------------------------------------------------------|------------------------------------------------|--------------------------|------------|--------|
| 1    | 14.743         | 1,2,3,5-Cyclohexanetetrol                                | C <sub>6</sub> H <sub>12</sub> O <sub>4</sub>  | 619                      | 53585-08-3 | 3.63   |
| 2    | 17.057         | Phytol, acetate                                          | C <sub>22</sub> H <sub>42</sub> O              | 339                      | 0-00-0     | 7.29   |
| 3    | 19.514         | n-Nonadecanol-1                                          | C <sub>19</sub> H <sub>40</sub> O              | 285                      | 1454-84-8  | 5.35   |
| 4    | 19.747         | Phytol                                                   | C <sub>20</sub> H <sub>40</sub> O              | 297                      | 150-86-7   | 4.66   |
| 5    | 21.140         | 1,2-15,16-Diepoxylhexadecane                             | C <sub>16</sub> H <sub>30</sub> O <sub>2</sub> | 254                      | 0-00-0     | 3.10   |
| 6    | 24.591         | 13-Docosenamide, (Z)                                     | C <sub>22</sub> H <sub>43</sub> NO             | 337                      | 112-84-5   | 2.46   |
| 7    | 24.897         | 1,4-Benzenedicarboxylic acid,<br>bis(2-ethylhexyl) ester | C <sub>24</sub> H <sub>38</sub> O <sub>4</sub> | 391                      | 6422-86-2  | 6.46   |
| 8    | 25.388         | Squalene                                                 | C <sub>30</sub> H <sub>50</sub>                | 410                      | 111-02-4   | 5.39   |
| 9    | 27.340         | 1-Heptacosanol                                           | C <sub>27</sub> H <sub>56</sub> O              | 397                      | 2004-39-9  | 4.27   |
| 10   | 27.579         | Vitamin E                                                | C <sub>29</sub> H <sub>50</sub> O <sub>2</sub> | 431                      | 59-02-9    | 3.67   |
| 11   | 27.723         | Flavone,<br>4',5-dihydroxy-6,7-dimethoxy-                | C <sub>17</sub> H <sub>14</sub> O <sub>6</sub> | 314                      | 6601-62-3  | 11.69  |
| 12   | 28.491         | Campesterol                                              | C <sub>28</sub> H <sub>48</sub> O              | 401                      | 474-62-4   | 5.16   |
| 13   | 28.705         | Stigmasterol                                             | C <sub>29</sub> H <sub>48</sub> O              | 413                      | 83-48-7    | 4.01   |
| 14   | 29.257         | Beta-Sitosterol                                          | C <sub>29</sub> H <sub>50</sub> O              | 415                      | 83-46-5    | 6.70   |
| 15   | 29.763         | Alpha-Amyrin                                             | C <sub>30</sub> H <sub>50</sub> O              | 427                      | 638-95-9   | 3.22   |
| 16   | 30.390         | Simiarenol                                               | C <sub>30</sub> H <sub>50</sub> O              | 427                      | 1615-94-7  | 4.25   |

**Table 7.** Phytochemical compounds identified in stem hexane extracts of *B. allostellata* by GC-MS analysis.

| Peak | Retention Time | Phytochemical Compound                                                                                           | Molecular Formula                                              | Molecular Weight | CAS No.   | Area % |
|------|----------------|------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|------------------|-----------|--------|
| 1    | 17.759         | Pentadecanoic acid                                                                                               | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>                 | 242              | 1002-84-2 | 1.00   |
| 2    | 19.373         | 9,12-Octadecadienoic acid (Z)                                                                                    | C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>                 | 280              | 60-33-3   | 2.33   |
| 3    | 19.433         | Dichloroacetic acid, tridec-2-ynyl ester                                                                         | C <sub>15</sub> H <sub>24</sub> C <sub>12</sub> O <sub>2</sub> | 306              | 0-00-0    | 1.52   |
| 4    | 19.646         | Octadecanoic acid                                                                                                | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>                 | 284              | 57-11-4   | 1.52   |
| 5    | 19.673         | Decanedioic acid, dibutyl ester                                                                                  | C <sub>18</sub> H <sub>34</sub> O <sub>4</sub>                 | 314              | 109-43-3  | 1.39   |
| 6    | 24.601         | 13-Docosenamide, (Z)                                                                                             | C <sub>22</sub> H <sub>43</sub> NO                             | 337              | 112-84-5  | 1.32   |
| 7    | 28.125         | Tetratetracontane                                                                                                | C <sub>44</sub> H <sub>90</sub>                                | 618              | 7098-22-8 | 1.45   |
| 8    | 28.796         | 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one | C <sub>30</sub> H <sub>48</sub> O                              | 424              | 0-00-0    | 2.35   |

**Table 8.** Phytochemical compounds identified in stem chloroform extracts of *B. allostellata* by GC-MS analysis.

| Peak | Retention Time | Phytochemical Compound                       | Molecular Formula                              | Molecular Weight | CAS No.   | Area % |
|------|----------------|----------------------------------------------|------------------------------------------------|------------------|-----------|--------|
| 1    | 12.711         | Phenol, 2,4-bis(1,1-dimethylethyl)-          | C <sub>14</sub> H <sub>22</sub> O              | 206              | 96-76-4   | 1.20   |
| 2    | 17.750         | Pentadecanoic acid                           | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub> | 242              | 1002-84-2 | 1.00   |
| 3    | 19.641         | Octadecanoic acid                            | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> | 284              | 57-11-4   | 1.06   |
| 4    | 19.675         | Decanedioic acid, dibutyl ester              | C <sub>18</sub> H <sub>34</sub> O <sub>4</sub> | 314              | 109-43-3  | 1.07   |
| 5    | 24.168         | Octadecanoic acid, 2,3-dihydroxypropyl ester | C <sub>21</sub> H <sub>42</sub> O <sub>4</sub> | 358              | 123-94-4  | 1.03   |
| 6    | 24.612         | 13-Docosenamide, (Z)                         | C <sub>22</sub> H <sub>43</sub> NO             | 337              | 112-84-5  | 1.21   |

**Table 9.** Phytochemical compounds identified in stem methanol extracts of *B. allostellata* by GC-MS analysis.

| Peak | Retention Time | Phytochemical Compound                                             | Molecular Formula                              | Molecular Weight | CAS No.   | Area % |
|------|----------------|--------------------------------------------------------------------|------------------------------------------------|------------------|-----------|--------|
| 1    | 19.514         | <i>n</i> -Nonadecanol-1                                            | C <sub>19</sub> H <sub>40</sub> O              | 285              | 1454-84-8 | 5.84   |
| 2    | 20.942         | Tributyl acetylcitrate                                             | C <sub>20</sub> H <sub>34</sub> O <sub>8</sub> | 402              | 77-90-7   | 1.05   |
| 3    | 21.139         | 1,2-15,16-Diepoxyhexadecane                                        | C <sub>16</sub> H <sub>30</sub> O <sub>2</sub> | 254              | 0-00-0    | 1.23   |
| 4    | 21.967         | 9-Octadecenamide                                                   | C <sub>18</sub> H <sub>35</sub> NO             | 281              | 301-02-0  | 2.19   |
| 5    | 24.588         | 13-Docosenamide, (Z)                                               | C <sub>22</sub> H <sub>43</sub> NO             | 337              | 112-84-5  | 1.14   |
| 6    | 24.901         | 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester              | C <sub>24</sub> H <sub>38</sub> O <sub>4</sub> | 391              | 6422-86-2 | 25.68  |
| 7    | 25.386         | Squalene                                                           | C <sub>30</sub> H <sub>50</sub>                | 410              | 111-02-4  | 1.39   |
| 8    | 27.337         | 1-Heptacosanol                                                     | C <sub>27</sub> H <sub>56</sub> O              | 397              | 2004-39-9 | 1.72   |
| 9    | 28.703         | Stigmasterol                                                       | C <sub>29</sub> H <sub>48</sub> O              | 413              | 83-48-7   | 1.89   |
| 10   | 29.252         | Beta-Sitosterol                                                    | C <sub>29</sub> H <sub>50</sub> O              | 415              | 83-46-5   | 5.68   |
| 11   | 29.453         | Alpha. Amyrenone                                                   | C <sub>30</sub> H <sub>48</sub> O              | 425              | 0-00-0    | 2.18   |
| 12   | 29.755         | Alpha-Amyrin                                                       | C <sub>30</sub> H <sub>50</sub> O              | 427              | 638-95-9  | 3.16   |
| 13   | 29.928         | Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,4,5,6,7,8 | C <sub>17</sub> H <sub>26</sub> O <sub>3</sub> | 278              | 0-00-0    | 2.01   |
| 14   | 30.195         | Stigmasta-3,5-dien-7-one                                           | C <sub>29</sub> H <sub>46</sub> O              | 411              | 2034-72-2 | 1.51   |
| 15   | 30.604         | Cholest-4-en-3-one                                                 | C <sub>27</sub> H <sub>44</sub> O              | 385              | 601-57-0  | 1.13   |

For the leaf hexane chromatogram, the highest peak identified was tetratetracontane, which had the highest percentage area of 3.25% (Table 4). This compound displays antioxidant, cytoprotective and anti-inflammatory activities [113,117]. The lowest peak identified in the leaf hexane (Table 4), stem hexane (Table 7), and stem chloroform (Table 8) chromatogram was pentadecanoic acid, with an area percentage of 1.02%, 1.00% and 1.00%, respectively. It should be noted that pentadecanoic acid has not been reported in any species of *Barleria*; however, this compound is a fatty acid and is found in the milk fat of cows, regulates hormones, improves the immune system and boosts metabolism [118,119].

Octadecanoic acid, 2,3-dihydroxypropyl ester and tetratetracontane displayed the lowest peaks in the leaf chloroform chromatogram, with an area percentage of 1.00% (Table 5). These compounds have not been reported in any species of *Barleria*, though octadecanoic acid, 2,3-dihydroxypropyl ester displays anticancer and antimicrobial activities (Table 10). In the leaf methanolic chromatogram, flavone, 4',5-dihydroxy-6,7-dimethoxy-exhibited the highest peak, with an area percentage of 11.69% (Table 6). The 13-docosenamide, (Z) displayed the lowest peak, with an average percentage of 2.46%, and has not been reported in any species of *Barleria* (Table 6) but was reported to exhibit antimicrobial properties in *Ludwigia perennis* [120].

The highest peak for the stem hexane chromatogram was 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one, which had an average percentage of 2.35 (Table 7). This compound was reported to exhibit antibacterial, antioxidant, antitumour and cancer preventives (Table 10). Additionally, the lowest peak on the stem methanol chromatogram was tributyl acetyl citrate (Table 7). Al-Rubaye et al. [121] examined the methanolic leaf extracts of *Sinapis arvensis* for its medicinal properties. These authors found tributyl acetyl citrate to display antioxidant and anti-inflammatory activities.

The identified compounds illustrated in Table 10, possessed various biological properties of medicinal importance. Several compounds found in the extracts of *B. albostellata* were also noted in other species of *Barleria*. Phyto-compounds such as phenol, 2,4-bis(1,1-dimethylethyl), found in *B. albostellata* (Table 10), were identified in *B. prionitis* [122], *B. montana* [123] and *B. lupulina* [124]. The 9,12,15-octadecatrienoic acid, (Z,Z,Z) (Table 10) was only prominent in *B. buxifolia* [125]. Kumari and Dubey [124] reported on octadecanoic acid (Table 10) in the extracts of *B. lupulina*, while Sriram and Sasikumar [123] found this compound in *B. montana*. Squalene found in *B. albostellata* (Table 10) was also identified in *B. montana* [126], *B. longiflora* [127], *B. courtallica* [128], *B. lupulina* [124] and *B. grandiflora* [129].

Eicosane, a solid n-alkane (Table 10), was found in extracts of *B. courtallica* [128], *B. prionitis* [130] and *B. dinteri* [10]. In the extracts of *B. courtallica* [128] and *B. lupulina* [124], phytol and acetate (Table 10) were identified. Furthermore, phytol (Table 10) was reported in *B. montana* [126], *B. longiflora* [127], *B. courtallica* [128], *B. lupulina* [124], *B. strigosa* [131], *B. buxifolia* [125] and *B. prionitis* [130]. Vitamin E (Table 10), a fat-soluble vitamin, was only noted in *B. courtallica* [128]. Flavones, a class of flavonoids, found in the extracts of *B. albostellata* (Table 10) were also reported in *B. prionitis* [132] and *B. acanthoides* [133].

Campesterol found in *B. longiflora* [134]; stigmasterol in *B. courtallica* [128], *B. montana* [123], *B. longiflora* [127], *B. cristata*, *B. prionitis* [135,136] and *B. lupulina* [137]; and beta-sitosterol identified in *B. prionitis* [130], *B. courtallica* [128], *B. montana* [126] and *B. longiflora* [127] are three characteristic phytosterols found in *B. albostellata* (Table 10). Stigmasta-3,5-dien-7-one has only been reported in *B. albostellata* (Table 10), while 13,14-seco-stigmasta-5,14-diene-3 $\alpha$ -o was noted in *B. prionitis* [138]. Additionally, alpha-amyrin was noted in *B. cristata* [135] and *B. prionitis* [130]. Sujatha et al. [128] and Kumari and Dubey [124] reported the presence of 9,12-octadecadienoic acid (Z,Z) (Table 10) in the extracts of *B. courtallica* and *B. lupulina*, respectively.

To date, 1-heptacosanol; 1-(+)-ascorbic acid 2,6-dihexadecanoate; tridecanoic acid; decanedioic acid, dibutyl ester; 1,2,3,5-cyclohexanetetrol; 1,2-15,16-diepoxyhexadecane; 1,4-benzenedicarboxylic acid, bis(2-ethylhexyl) ester; simiarenol; dichloroacetic acid, tridec-2-ynyl ester; 4,4,6a,6b,8a,11,11,14b-octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-



octadecahydro-2H-picen-3-one; alpha amyrenone; acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,4,5,6,7,8; and cholest-4-en-3-one, found in *B. albostellata*, were not reported in any species of *Barleria*. Although GC-MS analysis identified the phytochemical constituents present in the hexane, chloroform and methanolic extracts, it should be noted that the most compounds were found in the leaf (Table 6) and stem methanolic (Table 9) extracts.

**Table 10.** Pharmacological activities of compounds found in *B. albostellata*.

| No | Phytochemical Compound                                | Pharmacological Action                                                                                                                                                                   | References        |
|----|-------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| 1  | Pentadecanoic acid                                    | Flavouring agent, lubricants, adhesive agents, ability to regulate hormones, improve the immune system, boost metabolism and inhibit production of uric acid                             | [118,138–141]     |
| 2  | 9,12,15-Octadecatrienoic acid, (Z)                    | Antioxidant, anti-inflammatory, antimicrobial, diuretic, anticancer, antitumour, chemo-preventive properties used in vaccine formulations and reduced complications in COVID-19 patients | [142–145]         |
| 3  | Octadecanoic acid                                     | Antimicrobial activity                                                                                                                                                                   | [146–148]         |
| 4  | 13-Docosenamide, (Z)                                  | Antimicrobial activity                                                                                                                                                                   | [119,149]         |
| 5  | Squalene                                              | Cosmetics, skin ointments, antioxidant, antitumour, anticancer, chemo-preventive and sunscreen properties                                                                                | [150–152]         |
| 6  | Eicosane                                              | Antitumour, antifungal activity and bronchodilators                                                                                                                                      | [153–155]         |
| 7  | 1-Heptacosanol                                        | Nematicidal, anticancer, antioxidant and antimicrobial properties                                                                                                                        | [156–158]         |
| 8  | Tetratetracontane                                     | Plant growth production, antioxidant, cytoprotective and anti-inflammatory activities                                                                                                    | [113,138,159,160] |
| 9  | 1-(+)-Ascorbic acid 2,6-dihexadecanoate               | Antioxidant food additive, antimetastatic, anti-invasive, cancer, cardio protective and anti-infertility                                                                                 | [161–163]         |
| 10 | Tridecanoic acid                                      | Antifungal, antibacterial and larvicidal                                                                                                                                                 | [164–166]         |
| 11 | Decanedioic acid, dibutyl ester                       | Antimicrobial, antispasmodic and anti-inflammatory effects                                                                                                                               | [167]             |
| 12 | Octadecanoic acid, 2,3-dihydroxypropyl ester          | Anticancer, antimicrobial, acidifier, acidulant, arachidonic acid inhibitor and inhibits production of uric acid                                                                         | [139,141]         |
| 13 | 1,2,3,5-Cyclohexanetetrol                             | Antioxidant, antimicrobial and anti-inflammatory properties                                                                                                                              | [168]             |
| 14 | Phytol, acetate                                       | Anti-inflammatory, antileishmanial, anti-trypanosomal, antimicrobial, anticancer and diuretic                                                                                            | [32,169–171]      |
| 15 | <i>n</i> -Nonadecanol-1                               | Antimicrobial and cytotoxic properties                                                                                                                                                   | [172,173]         |
| 16 | Phytol                                                | Anticancer, antimicrobial, anti-inflammatory, antioxidant activity, diuretic, cosmetics and used in the fragrance industry                                                               | [141,174]         |
| 17 | 1,2-15,16-Diepoxyhexadecane                           | Antitumour and anti-inflammatory properties                                                                                                                                              | [175]             |
| 18 | 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | Anticancer properties                                                                                                                                                                    | [176,177]         |
| 19 | Vitamin E                                             | Skin repair, enhancing the immune system and has anticancer, antitumour and antioxidant properties                                                                                       | [151,178,179]     |
| 20 | Flavone                                               | Antibacterial, antimutagenic, antiviral and antioxidant activity                                                                                                                         | [180–182]         |

Table 10. Cont.

| No | Phytochemical Compound                                                                                           | Pharmacological Action                                                                                                                                                                                 | References    |
|----|------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| 21 | Campesterol                                                                                                      | Anti-inflammatory and anticancer activity                                                                                                                                                              | [183,184]     |
| 22 | Stigmasterol                                                                                                     | Anti-inflammatory, anti-asthma, anticancerous, anti-inflammatory, antiarthritic, hypoglycemic, antioxidant and thyroid-inhibiting properties, analgesic, antiosteoarthritic and antimutagenic activity | [141,185,186] |
| 23 | Beta-Sitosterol                                                                                                  | Reduces cholesterol levels, androgen blocker, anti-amyloid beta and anticancer properties                                                                                                              | [141,187]     |
| 24 | Alpha-Amyrin                                                                                                     | Alpha amylase and glucosidase inhibitor, antioxidant, antibacterial and anti-inflammatory properties                                                                                                   | [151,188]     |
| 25 | Simiarenol                                                                                                       | Antinociceptive activity                                                                                                                                                                               | [189]         |
| 26 | 9,12-Octadecadienoic acid (Z,Z)-                                                                                 | Anti-inflammatory, antibacterial, antiarthritic, hepatoprotective, anti-histaminic, anticoronary and anticancer properties                                                                             | [139,190,191] |
| 27 | Dichloroacetic acid, tridec-2-ynyl ester                                                                         | Cosmetic treatments, anticancer, antimicrobial, antioxidant activity                                                                                                                                   | [192,193]     |
| 28 | 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one | Antibacterial, antioxidant, antitumour and cancer preventives                                                                                                                                          | [194,195]     |
| 29 | Phenol, 2,4-bis(1,1-dimethylethyl)-                                                                              | Antibacterial and anti-inflammatory activities                                                                                                                                                         | [196]         |
| 30 | Tributyl acetylcitrate                                                                                           | Anticancer and antimicrobial activities                                                                                                                                                                | [197–199]     |
| 31 | 9-Octadecenamide                                                                                                 | Antimicrobial activity                                                                                                                                                                                 | [148]         |
| 32 | Alpha. Amyrenone                                                                                                 | Antibacterial and antimalarial activities                                                                                                                                                              | [200,201]     |
| 33 | Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,4,5,6,7,8                                               | Antimicrobial activity                                                                                                                                                                                 | [202,203]     |
| 34 | Stigmasta-3,5-dien-7-one                                                                                         | Anti-diabetic and anticancer properties, free-radical scavenging activity                                                                                                                              | [204–206]     |
| 35 | Cholest-4-en-3-one                                                                                               | Anti-obesity and an intestinal metabolite of cholesterol                                                                                                                                               | [207,208]     |

#### 2.4. Antibacterial Activity of Leaf and Stem Extracts of *B. allostellata*

The current interest in herbal plants as therapeutic agents has increased in several parts of the world. This is due to the ever-increasing occurrence of drug-resistant bacteria and the influx of new pathogenic bacterial strains. Active phytochemicals found in hexane, chloroform and methanolic extracts of *B. allostellata* were subjected to antibacterial assays. Various concentrations (3.125, 6.25, 12.25, 25, 50 and 100 mg/mL) were tested against the Gram-positive (*B. subtilis*, methicillin-resistant *S. aureus* and *S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. The zone of inhibition of the growth of bacteria was used to evaluate the antibacterial potential of the various extracts. Results presented in Table 11 of certain leaf and stem extracts showed significant inhibition compared to streptomycin and gentamicin (positive controls) (Table 11). The screening was done in triplicate with streptomycin (Gram-positive) and gentamicin (Gram-negative) used as the standard antibacterial positive controls, and 10% DMSO without plant extracts was used as the negative control. Clear zones of inhibition were observed in the leaf and stem crude extracts against the various strains. Significance was established at  $p < 0.05$  for

all concentrations and treatments. The various extracts and concentrations displayed a variable degree of bacterial growth against various bacterial strains.

**Table 11.** Antibacterial activity of crude extracts from leaves and stem of *B. allostellata* against human pathogenic strains (zone of inhibition, mm).

| Strain                                              | Concentration (mg/mL) | Extracts    |                 |               |              |                 |               | Positive Control (mg/mL) |              |
|-----------------------------------------------------|-----------------------|-------------|-----------------|---------------|--------------|-----------------|---------------|--------------------------|--------------|
|                                                     |                       | Leaf Hexane | Leaf Chloroform | Leaf Methanol | Stem Hexane  | Stem Chloroform | Stem Methanol | Leaf                     | Stem         |
| <i>B. subtilis</i> (ATCC 6633)                      | 3.125                 | R           | R               | R             | R            | R               | R             | 9.00 ± 1.00              | 11.00 ± 1.00 |
|                                                     | 6.25                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 12.5                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 25                    | R           | 8.33 ± 1.53     | 8.00 ± 1.00   | R            | 7.33 ± 0.58     | 8.00 ± 0.00   |                          |              |
|                                                     | 50                    | 7.67 ± 2.08 | 7.67 ± 0.58     | 9.33 ± 0.58   | 7.67 ± 2.08  | 8.00 ± 1.00     | 8.67 ± 0.58   |                          |              |
|                                                     | 100                   | 9.00 ± 3.46 | 7.00 ± 0.00     | 10.00 ± 2.00  | 8.67 ± 1.52  | 10.00 ± 3.61    | 9.67 ± 0.58   |                          |              |
| Methicillin-resistant <i>S. aureus</i> (ATCC 43300) | 3.125                 | R           | R               | R             | R            | R               | R             | 9.33 ± 0.58              | 9.00 ± 1.00  |
|                                                     | 6.25                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 12.5                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 25                    | R           | 8.67 ± 0.58     | R             | R            | R               | R             |                          |              |
|                                                     | 50                    | R           | 9.00 ± 0.00     | 8.67 ± 2.08   | R            | 7.33 ± 0.58     | 8.00 ± 1.00   |                          |              |
|                                                     | 100                   | R           | 10.00 ± 0.00    | 11.00 ± 2.65  | 10.67 ± 2.31 | 8.00 ± 1.00     | 9.00 ± 2.00   |                          |              |
| <i>S. aureus</i> (ATCC 25923)                       | 3.125                 | R           | R               | R             | R            | R               | R             | 9.67 ± 0.58              | 10.00 ± 1.00 |
|                                                     | 6.25                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 12.5                  | R           | R               | 7.33 ± 0.58   | R            | R               | 7.00 ± 0.00   |                          |              |
|                                                     | 25                    | R           | 7.67 ± 0.58     | 8.00 ± 0.00   | 8.00 ± 1.00  | 7.33 ± 0.58     | 8.00 ± 1.00   |                          |              |
|                                                     | 50                    | 7.33 ± 0.58 | 8.67 ± 0.58     | 8.67 ± 1.53   | 9.00 ± 0.00  | 8.33 ± 0.58     | 10.00 ± 1.73  |                          |              |
|                                                     | 100                   | 9.33 ± 0.58 | 9.33 ± 0.58     | 10.33 ± 1.53  | 10.33 ± 1.53 | 9.00 ± 1.73     | 11.00 ± 2.65  |                          |              |
| <i>E. coli</i> (ATCC 35218)                         | 3.125                 | R           | R               | R             | R            | R               | R             | 8.67 ± 0.58              | 9.33 ± 0.58  |
|                                                     | 6.25                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 12.5                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 25                    | R           | 9.67 ± 0.58     | R             | R            | 9.33 ± 0.58     | R             |                          |              |
|                                                     | 50                    | R           | 10.67 ± 1.15    | 9.00 ± 3.46   | R            | 10.00 ± 1.00    | 9.67 ± 2.08   |                          |              |
|                                                     | 100                   | R           | 12.33 ± 2.08    | 12.67 ± 0.58  | R            | 11.33 ± 1.15    | 11.33 ± 1.15  |                          |              |
| <i>P. aeruginosa</i> (ATCC 25783)                   | 3.125                 | R           | R               | R             | R            | R               | R             | 9.33 ± 0.58              | 8.67 ± 1.15  |
|                                                     | 6.25                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 12.5                  | R           | R               | R             | R            | R               | R             |                          |              |
|                                                     | 25                    | R           | 8.67 ± 1.53     | 7.33 ± 0.58   | R            | 7.00 ± 0.00     | 9.33 ± 1.15   |                          |              |
|                                                     | 50                    | R           | 9.00 ± 0.00     | 8.67 ± 1.52   | R            | 8.67 ± 0.58     | 10.67 ± 2.87  |                          |              |
|                                                     | 100                   | R           | 10.00 ± 3.00    | 14.33 ± 1.53  | R            | 9.67 ± 1.15     | 12.33 ± 0.58  |                          |              |

R-resistant, positive controls (streptomycin 10 mg/mL (Gram-positive bacteria), gentamicin 10 mg/mL (Gram-negative bacteria)).

As the concentration increased (3.125, 6.25, 12.25, 25, 50 and 100 mg/mL), the zone of inhibition against various bacterial strains also increased. Inhibition against the various bacterial strains for the various extracts was noted at concentrations > 25 mg/mL. The highest inhibitory activity was observed at 100 mg/mL for both leaf and stem extracts for *B. subtilis* and *S. aureus*. MRSA, *E. coli* and *P. aeruginosa* were resistant to the leaf hexane extracts, whilst the stem hexane extracts displayed no inhibition against *E. coli* and *P. aeruginosa* only (Table 11). Gram-positive and Gram-negative bacteria were resistant to all extracts at both 3.125 and 6.25 mg/mL concentrations. Amoo et al. [57] verified the antibacterial activity of *B. allostellata* against *B. subtilis*, *S. aureus* and *E. coli*. However, low activity was observed against Gram-negative bacteria [57]. Matu and Van Staden [209] suggested that a thick murein layer present in the structure of Gram-negative bacteria may prevent the entry of inhibitors. The differences in the bacterial inhibition varied for each crude extract. The leaf methanolic extracts at 100 mg/mL displayed the highest inhibition against all tested bacterial strains. However, the stem methanolic extracts had the highest inhibition against *S. aureus* and *P. aeruginosa* only.

Several notable bioactive compounds found in the leaf and stem extracts of *B. allostellata* using GC-MS analysis were reported to display antibacterial efficiency. The presence of phytol and flavone found in the leaf methanolic extracts of *B. allostellata* could be responsible for the antibacterial effects against the several tested strains. Phytol was reported to severely damage the deoxyribonucleic acid (DNA) of bacteria by inducing oxidative stress [210]. The presence of flavonoids blocks important enzymes that play a significant role in the reproduction, growth, cell rupture or functional modification in

bacteria [211]. Stigmasterol, another compound found in the leaf methanolic extract, was reported to act as a lactamase inhibitor, which prevented antibacterial resistance by restoring the vulnerability of the antibiotic resistant bacteria to antibiotics [212]. The mode of action used by most bioactive compounds in treating microbial infections is by interacting with the microbial enzyme system, interfering with nucleic acids, the cell wall and the cell membrane [213–215].

Additionally, the antibacterial efficiency in the various extracts may be due to greater solubility of phyto-compounds in polar solvents than non-polar solvents [123]. It was recommended that the inability of plant extracts of other solvent systems to display antibacterial activity against the various bacterial strains could be due to these strains exhibiting some sort of resistance mechanism, e.g., alteration of target sites, enzyme inactivation, reduced drug accumulation or the amount of bioactive compounds present is very low [216]. Extracts of *B. acuminata* [69], *B. cristata* [217], *B. greenii* [57], *B. prionitis* [218] and *B. montana* [125] exhibited antibacterial activity against *B. subtilis* and *S. aureus*. However, *B. cristata* displayed low inhibition against *E. coli* [217], and *B. montana* [125] displayed moderate activity against *E. coli* and *P. aeruginosa*. According to Kumari and Dubey [123], ethanolic leaf extracts of *B. lupulina* inhibited the growth of *E. coli*, *S. aureus* and *P. aeruginosa*, whereas methanolic extracts displayed zones of inhibition against *S. aureus* and no inhibition against *E. coli* and *P. aeruginosa* [219]. Various medicinal plant extracts were reported to display greater activity against Gram-positive bacteria as opposed to Gram-negative bacteria [57,220,221]. The positive results in the present study could be attributed to the location of collected plant material, active constituents found in the different extracts, various extraction preparation, the broad range of treatment concentrations and the variety of bacterial strains. Therefore, the active constituents found in the different extracts of this plant were effective against both Gram-positive and -negative bacteria.

Qualitative phytochemical screening and GC-MS revealed various biologically active compounds which have been known to contain diverse activities that may help protect against chronic diseases [66,67]. Several compounds found in the extracts of *B. allostellata* were also noted in other species of *Barleria* [124,131,137,138,140,141]. Additionally, the phyto-constituents found in the leaf and stem crude extracts could inhibit the growth of various pathogenic strains. Various medicinal plant extracts were reported to display greater activity against Gram-positive bacteria as opposed to Gram-negative bacteria [57,220,221]. The antibacterial efficiency in the various extracts may be due to greater solubility of phyto-compounds in polar solvents than non-polar solvents [136].

### 3. Materials and Methods

#### 3.1. Plant Materials

Leaves and stems of *B. allostellata* were collected from the University of KwaZulu-Natal, Westville campus (29°49'51.6" S, 30°55'30" E), Durban, South Africa. A voucher specimen (7973000) was deposited in the Ward Herbarium of the University of KwaZulu-Natal, Life Sciences, Westville campus.

#### 3.2. Organoleptic Evaluation

The evaluation of crude leaf and stem material was completed with the aid of sensory organs following standard methods [221]. This protocol uses colour, odour, taste and texture. Organoleptic assessment is accomplished using organs of sense and describing specific features of the material. This assessment is regarded as a first step towards establishing the identity and degree of purity of the sample [222].

#### 3.3. Preparation of Crude Extract

For the preparation of the crude extract, leaves and stems were oven-dried for 2 weeks at 35°. The dried materials were crushed to a fine powder with the aid of a mechanical blender (Russel Hobbs, model: RHB315). The powdered material underwent sequential extraction using various solvents (hexane, chloroform and methanol) in a Soxhlet apparatus.

Approximately 10 g of powdered leaves were placed into a round-bottom flask containing 100 mL of hexane, the appropriate solvent, and boiled for 3 h at 40 °C. The extracted solution was filtered (Whatman® No. 1 filter paper) and retained. This procedure was conducted in replicates. Consecutive extractions of chloroform followed by methanol were achieved. Each solvent extraction followed the same process as mentioned above. Successive extractions were performed on the leaf and stem material.

#### Evaporation and Concentration

The concentration of each extract was left to evaporate in a dark fume-hood, at room temperature. The dried extracts were stored in airtight, labelled glass jars to prevent the material from reacting with the atmospheric humidity. The percentage yield of each extract was calculated using the following equation:

$$\text{Extract Yield (\%)} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of plant material (g)}} \times 100$$

#### 3.4. Qualitative Phytochemical Analysis

Preliminary phytochemical screening was carried out on the powdered leaf and stem material and chemically tested for the presence of various constituents using standard protocols [223–226].

#### 3.5. Fluorescence Analysis

Fluorescent analysis of the dried powdered plant material plays an important role in the determining the quality and purity of the tested drug. A small quantity of the dry plant powder (leaves and stems) was placed separately onto clean microscope slides. Two drops of each prepared reagent were dispensed, mixed gently by slanting the slide and allowed to stand for 3 min for the thorough absorption of the solution by the plant powder. The slides were then viewed using a Nikon Eclipse microscope, using bright field light and UV-2A (excitation 320/380) illumination. The colours attained by the application of various reagents were recorded. Fluorescence analysis of the leaf and stem powder was carried out using the standard method [227,228].

#### 3.6. Gas Chromatography-Mass Spectrometry (GC-MS)

This analysis is used to examine liquid, gaseous or solid samples and produce several different peaks in the gas chromatogram. Each peak generates a specific mass spectrum which is used for compound identification. Leaf and stem methanolic extracts were analysed using the GC-MS (QP-2010 Ultra SE, Shimadzu, Kyoto, Japan) instrument, with an Rx\_5Sil Ms capillary column (0.25 µm internal diameter and 0.25 µm film thickness) from Restek (Bellefonte, PA, USA). The carrier gas, helium, had a flow rate of 0.96 mL/min, a total flow of 4.9 mL/min and a linear velocity of 36.7 cm/sec at a purge flow of 3.0 mL/min. The injection temperature was set at 250 °C. The oven temperature was set at 50 °C and held for 1 min, increased to 310 °C and held for a further 10 min. Chemical compounds (analytes) were identified by relating their retention times with those of the polychlorinated biphenyl (PCB) standards found in the National Institute of Standards and Technology (NIST) library. This analysis was conducted at the Department of Chemistry at the University of KwaZulu-Natal, Westville campus.

#### 3.7. Antibacterial Bioassay

Crude (hexane, chloroform and methanol) leaf and stem extracts were transferred to Eppendorf centrifuge tubes, dissolved in 10% dimethyl sulfoxide (DMSO) at various concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL and homogenised using a vortex. The prepared sample was stored at −4 °C until further use. The prepared crude extracts were subjected to antibacterial assays. Leaf and stem samples were tested against Gram-positive bacteria (*Bacillus subtilus* ATCC 6633, methicillin-resistant *Staphylococcus*



*aureus* ATCC 43300 and *Staphylococcus aureus* ATCC 25923) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 25783 and *Escherichia coli* ATCC 35218). These bacterial strains were supplied by Professor Johnson Lin, School of Life Sciences (Microbiology Department), University of KwaZulu-Natal, and maintained in 75% glycerol at  $-80\text{ }^{\circ}\text{C}$  before the experiment was conducted.

In vitro antibacterial screening of the prepared extracts was conducted using the agar disc diffusion technique as per the Clinical and Laboratory Standards Institute (CLSI) guidelines [229]. Both Gram-positive and Gram-negative bacteria from stock cultures were sub-cultured onto fresh agar plates and incubated overnight at  $37\text{ }^{\circ}\text{C}$ . Glass test tubes containing distilled water (15–20 mL) were autoclaved at  $121\text{ }^{\circ}\text{C}$  for 1 h. Colonies of bacteria from each Petri plate were harvested with a sterile loop and inoculated by transferring a loopful into glass test tubes containing 15 mL of sterile distilled water (0.5 McFarland scale). The absorbance of each bacterial culture was measured, adjusted and diluted to attain a viable cell count using the Cary 60 UV-Vis spectrophotometer.

Each bacterial strain was separately smeared uniformly over the surface of the Mueller–Hinton agar plates with a sterile cotton swab. Sterile Whatman filter paper No. 1 discs (diameter 6 mm) were impregnated with 20  $\mu\text{L}$  of the respective extract concentrations (3.125, 6.25, 12.5, 25, 50, 100 mg/mL) and dried at room temperature for 1 h before use [230]. The prepared sterile discs containing extracts were placed carefully onto the agar using sterile forceps. Petri plates were sealed and incubated for 24 h at  $37\text{ }^{\circ}\text{C}$ . Zones of inhibition evident around the filter paper were taken as positive results. The diameters of inhibition were measured and photographed within 18–24 h after incubation to determine if the extract exhibited any antibacterial activity. Filter paper discs loaded with streptomycin and gentamycin were used as positive controls and 10% DMSO as the negative control [231]. The analyses were conducted in triplicate, and data were presented as mean  $\pm$  standard deviation.

### 3.8. Statistical Analysis

All experiments conducted for the antibacterial assay were carried out in triplicate. Values were expressed as mean  $\pm$  standard deviation (significant at  $p < 0.05$  level). Antibacterial data were statistically analysed using the one-way analysis of variance (ANOVA).

## 4. Conclusions

It is evident from the present study that the qualitative colour tests, fluorescence and GC-MS analysis that the leaves and stems of *B. albostellata* possess biologically active compounds. Important compounds identified in leaf and stem extracts of *B. albostellata* were alkaloids, flavonoids and phenols. These compounds are known to display several diverse activities that may help protect against chronic diseases. Major pharmacologically active compounds found in the extracts were alpha-amyrin, flavone, phenol, phytol, phytol acetate, squalene and stigmasterol. Additionally, phyto-constituents found in the hexane, chloroform and methanol leaf and stem extracts of *B. albostellata* could inhibit the growth of various pathogenic strains ( $p < 0.05$ ). Other solvents such as ethanol and acetone can be used in extracting phytochemical compounds from the leaves and stems. These extractions can be subjected to antibacterial assays in order to evaluate its potency against various pathogenic strains. Further studies should be conducted on the isolation, identification and characterisation of the bioactive compounds in *B. albostellata* that may be responsible for its bioactivity. This is important to further understand the mechanisms involved in the antibacterial activity. The bioactive compounds and pharmacological activities of *B. albostellata* will provide a basic understanding of the importance of this species as a medicinal plant and a potential source for novel and useful drugs. Additionally, other parts of the plant such as the flowers and roots should be assessed for their safety and bioactivity and to identify any new therapeutic compounds or drug leads.

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## Review

# Essential Oils and Extracts from Epazote (*Dysphania ambrosioides*): A Phytochemical Treasure with Multiple Applications

Arsenio Heredia Severino <sup>1,2</sup>, Juana Fernández-López <sup>3</sup>, Fernando Borrás-Rocher <sup>4</sup> and Manuel Viuda-Martos <sup>3,\*</sup>

<sup>1</sup> Facultad de Ciencias Agronómicas y Veterinarias, Universidad Autónoma de Santo Domingo-UASD, Santo Domingo 10105, Dominican Republic; bioarsenio@gmail.com

<sup>2</sup> Instituto Dominicano de Investigaciones Agropecuarias y Forestales-IDIAF, Santo Domingo 10205, Dominican Republic

<sup>3</sup> IPOA Research Group, Institute on Agri-Food and Agri-Environmental Research and Innovation (CIAGRO-UMH), Miguel Hernández University, 03312 Orihuela, Spain; j.fernandez@umh.es

<sup>4</sup> Statistics and Operative Research Department, Miguel Hernández University, 03202 Elche, Spain; f.borras@umh.es

\* Correspondence: mviuda@umh.es; Tel.: +34-96-6749661

**Abstract:** *Dysphania ambrosioides*, commonly known as epazote, is a medicinal plant of great relevance in traditional Latin American medicine. Its cultural roots and pharmacological properties have made it an object of study for phytochemical research. An artificial intelligence (AI) tool was utilized to assist in reviewing scientific information regarding *D. ambrosioides*. An initial search was conducted in the Scopus database using the keywords epazote, *D. ambrosioides*, anti-helminthic, antioxidant, and antimicrobial, which yielded a total of 814 publications. To select the most relevant articles, this AI tool based on natural language processing (available online and free of charge) was applied, which analyzed the keywords that appeared in the titles and abstracts of the works and clustered them, leading to a reduction of 86.73% in the number of studies. *D. ambrosioides* stands out for its rich composition of bioactive compounds, which give the plant a wide range of therapeutic properties, including antiparasitic activity, through which it is effective against several parasites, such as helminths and protozoa, due to its schistosomicidal, nematocidal and antimalarial action. Additionally, it has shown antimicrobial, antioxidant, and anticancer properties as it contains compounds that help fight cell damage caused by free radicals. Epazote represents a rich source of compounds with a wide therapeutic range. However, much research is required to understand the mechanisms of action of these compounds and to evaluate their safety and efficacy in clinical trials.

**Keywords:** *Dysphania ambrosioides*; epazote; medicinal plants; bioactive compounds; pharmacological activity; phytotherapy; *Chenopodium ambrosioides*

## 1. Introduction

The *Dysphania ambrosioides* (L.) Mosyakin & Clemants, otherwise known as Jesuit's tea, Mexican tea, payqu (paico), epazote, mastruz, or herba sanctæ Mariæ (Arabic: M'khinza, French: anserine vermifuge) [1], is a wild species from tropical America, naturalized in North America, Africa, Europe, Australia, and Asian countries [2]. It is an herbaceous plant of the genus *Dysphania*, belonging to the family *Amaranthaceae* [3].

This plant (Figure 1) reaches up to 1 m in height, and it shows annual or perennial growth. It features stems that may be single or branched, and its leaves grow in an alternating pattern along the stem [4]. These leaves ranged from narrowly elliptic to elliptic and are green, oblong–lanceolate with the smallest leaves located at the top of the plant, directly attached to the stem (sessile), while the largest leaves are found at the base of the plant and have short petioles (leaf stalks). The flowers are small and green with dense terminal panicles of glomeruli, with five sepals. The plant is known for its strong and distinctive aroma. Its fruits and seeds are black and horizontal and are enclosed in a persistent calyx that is usually less than 0.8 mm long [5,6].

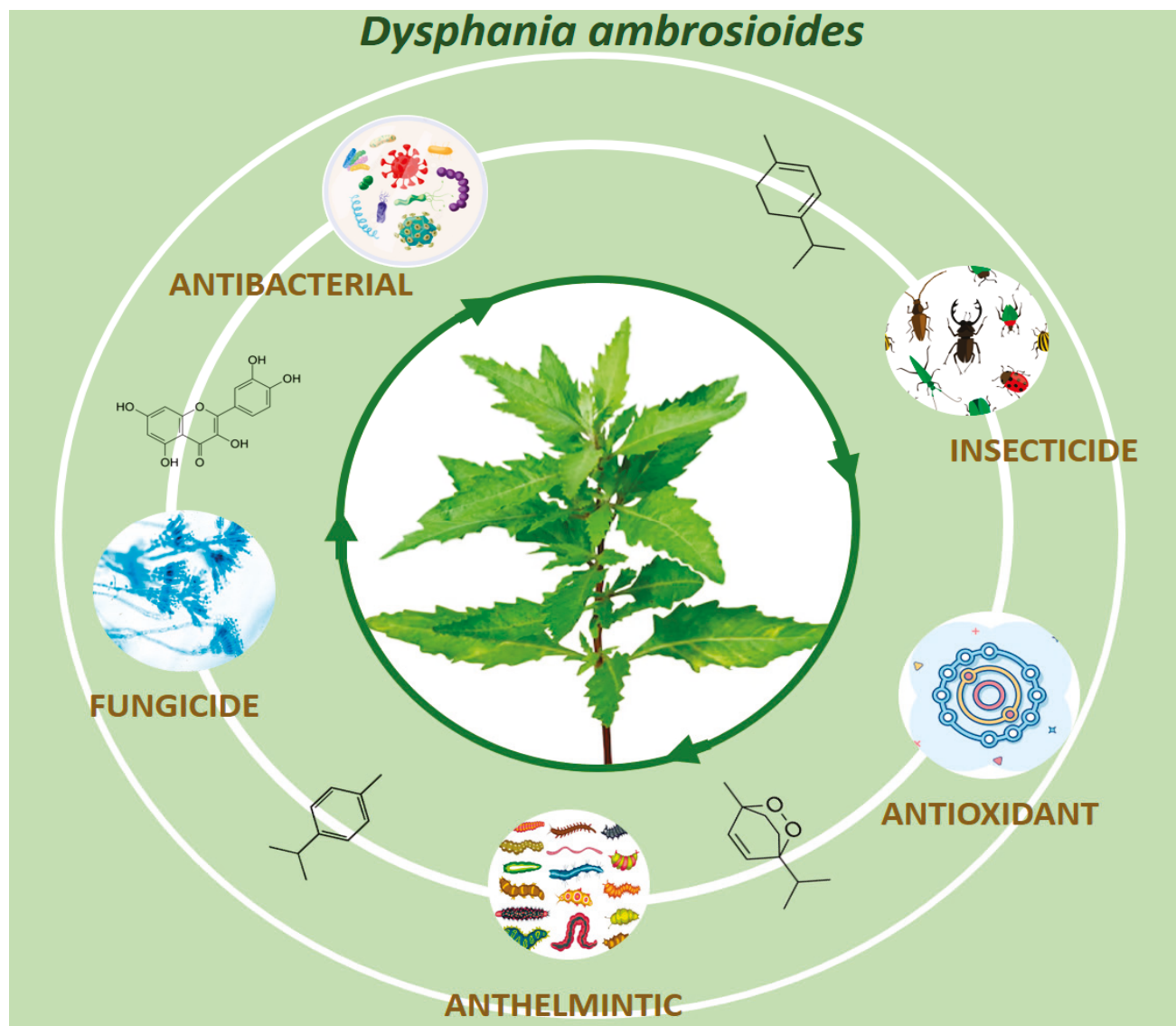


**Figure 1.** Different parts of the plant *Dysphania ambrosioides*.

The World Health Organization (WHO) reported that *D. ambrosioides* is among the most widely used medicinal plants around the world [1]. In Latin America, this plant is used in folk medicine because it has anti-helminthic, vermifuge, antispasmodic, and antipyretic activity. Additionally, it is used in the treatment of dental and digestive ailments, as well as in skin disorders, including dermal wounds and eczema [7–9]. In addition, the decoction, infusion, and maceration of leaves, stems, and branches were used in the treatment of respiratory disorders, cough, expectorant, and musculoskeletal injury [10]. In the Dominican Republic, the epazote is a shrubby plant that grows in the yard sporadically and has been used for the treatment of intestinal worms for centuries. In addition, this plant and its extracts have demonstrated several interesting biological activities such as antifungal, antioxidant, antibacterial, insecticide, and anti-helminthic effects (Figure 2). Other biological activities include anti-inflammatory activity, inhibiting cell proliferation, suppressing tumor growth, preventing cancer development, and inducing programmed cell death [11,12].

The use of plants for the treatment of diseases is nearly as ancient as the human species itself. They produce a diverse array of secondary metabolites, including vitamins, tannins, polyphenols, alkaloids, terpenoids, and flavonoids, among others [13], which exhibit useful biological activities such as antimicrobial, antiparasitic, and antioxidant activities. However, it is essential to conduct a phytochemical characterization to identify the biological activity of each active compound, as this will elucidate their respective advantages and disadvantages. On the other hand, there is an increasing interest in

developing natural and environmentally friendly derived alternatives for the control of insect pests, bacteria, parasites, and fungi [14]. So, plant derivatives represent a potential alternative in this context, being currently at the forefront of novel and promising drug development [15].



**Figure 2.** Biological activities of *Dysphania ambrosioides*.

Scientists often face the challenge of conducting literature reviews on topics that encompass hundreds or even thousands of articles. In these cases, the time and effort required to conduct it correctly can be overwhelming. Additionally, the process can often be influenced by the authors' backgrounds and knowledge, which can introduce selection bias and result in varying versions of the state of the art depending on the individual researchers. Trying to address this issue more efficiently and objectively, this study applies an easily accessible technological tool (available for free) based on artificial intelligence (AI) and natural language processing (NLP), which has been successfully applied in several literature review processes on topics related to food science [16] and biological activity [17]. This tool enables users to compare and analyze the state of the art in a more structured and objective manner, leading to a more reliable review process.

This review aims to summarize the current knowledge on the use of the *Dysphania ambrosioides* plant, highlighting its chemical composition, antibacterial, antifungal, antioxidant, antiparasitic, and insecticidal activity, as well as its potential applications. For the selection of the relevant information to be deeply studied and analyzed, an AI-based program was used.

## 2. Methodology

### 2.1. Bibliographic Search Conditions

This review was based on a bibliographic search using the SCOPUS and Web of Science databases that offers a comprehensive overview of global interdisciplinary scientific information, covering the areas of science, technology, and medicine (among others) in which the specific topic under study is framed. From this search, information about the titles of the papers, year of publication, abstract, and keywords was obtained. For this basic search, the following search conditions were selected: “epazote”, “Dysphania”, “ambrosioides”, and “Mexican Tea as the search topic; “review” and “article” as the document type; and “from 2015 to 2024” as the limit period for the search. No other filter or search restriction was applied. From this basic search, a file with 814 documents was obtained. From these documents, the search provided information on the titles of the articles, their year of publication, their abstracts, and keywords. This huge amount of information would require a highly time-consuming process to select a reasonable number of relevant papers to review in depth and obtain the required information about the aim of this review. To avoid that, it was decided to apply a new AI tool based on the NLP [16,17].

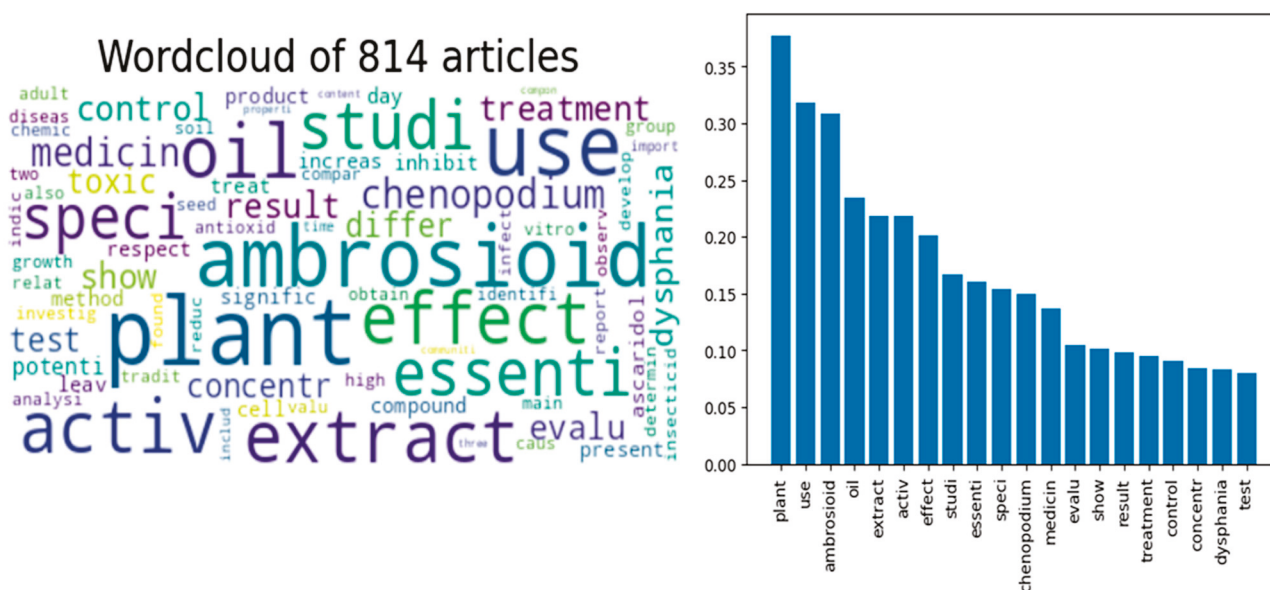
### 2.2. Selection Process of Relevant Papers Assisted by the AI Tool

This new AI tool has been specifically developed to assist in this process of selecting relevant information. The Jupiter notebook on Google Colab in this tool is freely available (Table S1) and has already been successfully applied with the same objective for a biology-related topic [17] and a food-related topic [16]. This new tool automatically assesses and categorizes all the documents by examining the content of their titles and abstracts, thus enabling the clustering of such a huge amount of bibliographic information [18]. Briefly, the operation of such an algorithm includes the steps described below.

#### 2.2.1. Preprocessing of Search Data

The data from the Scopus and Web of Science files required preprocessing before analysis to convert unstructured text data into normalized and structured data using the Python (version 3.12 for windows) Natural Language Toolkit library, which involved 3 steps: noise removal, normalization, and tokenization. After that, exploratory analysis tools were used to aggregate and visualize the text data associated with the 814 contributions obtained from the initial search (which lasted a total of 200 s), providing a bar plot and word cloud as a visual representation of the most frequently occurring words (75 words) in the abstracts used (Figure 3), which was very useful for selecting the most relevant articles. These graphical representations allowed for an understanding of the data to verify the preprocessing stage, ensuring that the analysis was on the right track, or alternatively, determining whether further preprocessing would be necessary before training the model.





**Figure 3.** Word cloud of the 75 most common words from the 814 papers about *Dysphania ambrosioides* and the corresponding frequency bar chart.

### 2.2.2. Text Clustering and Cluster Analyses

For text clustering, the tool uses the k-means algorithm, a simple and popular unsupervised clustering algorithm [19], in order to find groups of similar abstracts in the initial search file. This algorithm measures the distance between points (in this case, the abstracts of articles) and groups those that are close together, signifying their similarity. An effective cluster is characterized by having smaller distances between its points compared to the distances between points in separate clusters. Determining the optimal number of clusters is essential for organizing the information. To achieve this, two primary methods were employed: the elbow method and the average silhouette method [18–20]. In addition, this tool offers insightful details about the resulting clusters, including their relationships, proximity, temporal distribution, and other relevant aspects, the analysis of which allowed us to evaluate the resulting categorization or clustering and select the clusters that were considered to best represent the initial objective of the proposed review. This process can be repeated as many times as necessary to obtain a reasonable number of articles that will then be subjected to the traditional review process.

## 3. Selection of the Relevant Papers Assisted by the AI Tool

The optimal number of clusters obtained in the first analysis was 19 (numbered from 0 to 18). In this case, for the 19 clusters obtained, the information provided was the number of papers associated with each cluster (Figure S1); the corresponding word clouds (Figure S2) and figure bars (Figure S3) of the top words of each cluster; the results of the principal component analysis showing the relationship among the 19 clusters (Figure S4); and the distribution of the 19 clusters over the time (Figure S5).

From the analysis of all this information, clusters 0 (17 papers), 1 (79 papers), 3 (40 papers), 6 (60 papers), 7 (42 papers), 10 (68 papers), and 15 (96 papers) were selected as the most relevant for the study, mainly based on the analysis of the top words (Figures S2 and S3). In all of them, the words “extract”, “plant”, “activity”, “nematode”, “infection”, “insecticide”, “oil”, “essential”, or “compound” appeared as top words. By contrast, clusters in which words such as “seed”, “germination”, “infusion”, “volatiles”, “medicine”, “family”, “food”, “soil”, “tolerance”, “nanoparticles”, “metal”, “toxicity”,



“leishmaniosis” or “temperature” appeared as top words were not selected because they were not related to the aim of the present work. So, a total of 402 papers were included in the selected clusters, representing a reduction of 51% compared to the number of papers resulting from the initial search (814 papers). Nevertheless, it was still an unmanageable number of articles, so the initial process was repeated on these 402 papers (Table S1; second round). In this case, 19 subclusters were again obtained (also providing the same information as in the first round) (Table S1). In this second round, and from the analysis of these 19 subclusters, 3 of them were selected as relevant: subcluster 4 (71 papers included), subcluster 6 (76 papers included), and subcluster 14 (36 papers included). So, in this second round, a total of 179 documents were selected as relevant, indicating a total reduction of 78% compared to the initial search (814 papers). The word cloud of the top words included in each of these three subclusters is shown in Figure 2. It can be observed that the top words in these subclusters are “antimicrobial”, “antibacterial”, “antioxidant”, “insecticide”, “nematode”, “activity”, “control”, “extract”, “essential”, “oil”, “plant”, and “*ambrosioides*”, all of them highly related with the aim of this review. The number of papers included in these three subclusters (179) was considered feasible and allowed us to proceed with a traditional review, consisting of reading each paper in depth and extracting the most relevant results.

In the process of downloading documents, 32 documents were eliminated because they were repeated or they were not available as a publication in line and it was not able to obtain the full text. Therefore, the final number of papers that were downloaded and analyzed was 147. Of these, 23 papers were still discarded because they did not provide useful information for this study, so the final papers number included in this review was 124.

## 4. Traditional Review of the Selected Papers

### 4.1. Chemical Composition of *Dysphania ambrosioides*

The essential oils obtained from the different parts (leaves, stems, roots, and flowers) of *D. ambrosioides*, a species extensively used in traditional medicine in Costa Rica, were extensively analyzed, using several methodologies, including gas chromatography–mass spectrometry, gas chromatography–mass spectrometry tandem, and gas chromatography FID. The main components of essential oil obtained from this plant can be classified as monoterpenes and sesquiterpenes (as hydrocarbons, alcohols, ketones, etc., which may be acyclic, monocyclic, bicyclics, or tricyclics) [21]. In the same way, it is also possible to obtain ethanolic or methanolic extracts with a high content of bioactive compounds, mainly phenolic acids and flavonoids [6]. In the scientific literature, there were several studies where the chemical composition of *D. ambrosioides* essential oil had been determined, as shown in Table 1 [22–46]. Nevertheless, it should be noted that the composition and concentration of compounds depend on several factors, such as the part of the plant used, the methodology used to obtain the extracts, the environmental conditions, and the harvest period, as well as genetic factors [47]. In this sense, Fdil et al. [48] identified *p*-cymene (41.7%),  $\alpha$ -terpinene (34.8%), and ascaridole (10.8%) as major constituents of the essential oil obtained from leaves of *D. ambrosioides* collected in Morocco. Fatokun et al. [5] reported that the essential oil extracted from fresh leaves of *D. ambrosioides* by hydrodistillation had a total of twenty compounds with  $\gamma$ -terpinene (48.68%), *o*-cymene (21.71%), *trans*- $\beta$ -terpinyl butanoate (17.15%), and ascaridole (5.67%), which were the major compounds identified. Kandsi et al. [32] determined the composition of essential oils obtained by hydrodistillation of the aerial components of *D. ambrosioides* growing in Eastern Morocco. These authors reported the most abundant compounds in essential oil are (+)-4-carene (50.5%),  $\alpha$ -cyclogeraniol acetate (22.64%), and (1R,2R,3R,5S)-(–)-isopinocampheol (18.87%), respectively.

**Table 1.** Chemical composition of essential oils obtained from different parts of *Dysphania ambrosioides*.

| Extracts      | Part of the Plant | Compound                                                                                  | Concentration (%)                | References |
|---------------|-------------------|-------------------------------------------------------------------------------------------|----------------------------------|------------|
| Essential oil | Leaves            | Ascaridole-glycol<br>Linalool acetate<br>Dihydrocitronellol acetate                       | 10.58<br>11.26<br>19.53          | [22]       |
| Essential oil | Leaves            | $\alpha$ -Terpinene<br><i>p</i> -Cymene<br>Carvacrol<br>Ascaridole                        | 30.50<br>17.30<br>16.20<br>15.10 | [23]       |
| Essential oil | Leaves            | <i>Cis</i> -Piperitone oxide<br><i>Trans</i> -Isoascaridole<br><i>p</i> -Cymene           | 30.30<br>18.20<br>13.20          | [24]       |
| Essential oil | Leaves            | $\beta$ -Cymene<br>5-Isopropyl-6-methyl-hepta-3,5-dien-2-ol                               | 47.10<br>19.20                   | [25]       |
| Essential oil | Leaves and Stems  | $\alpha$ -Terpinene<br><i>p</i> -Cymene<br><i>Trans</i> -Chrysanthenyl acetate            | 59.7<br>22.8<br>13.8             | [26]       |
| Essential oil | Leaves            | Ascaridole<br><i>p</i> -Cymene                                                            | 49.77<br>42.32                   | [27]       |
| Essential oil | Leaves            | Ascaridole<br>$\alpha$ -Terpinene<br>Ascaridole epoxide                                   | 15.13<br>54.09<br>9.77           | [28]       |
| Essential oil | Leaves            | Ascaridole<br><i>p</i> -Cymene                                                            | 35.50<br>47.20                   | [7]        |
| Essential oil | Aerial Parts      | $\alpha$ -Terpinene<br>Ascaridole<br><i>p</i> -Cymene                                     | 53.4<br>17.7<br>12.1             | [46]       |
| Essential oil | Aerial Parts      | <i>p</i> -Cymene<br>4-Carene<br>$\alpha$ -Cyclogeraniol acetate                           | 31.72<br>27.34<br>16.90          | [30]       |
| Essential oil | Aerial Parts      | $\alpha$ -Terpinene<br><i>p</i> -Cymene                                                   | 72.5<br>20.6                     | [31]       |
| Essential oil | Aerial Parts      | 4-Carene<br>$\alpha$ -Cyclogeraniol acetate<br><i>Trans</i> - $\beta$ -Terpinyl butanoate | 50.50<br>22.64<br>31.13          | [32]       |
| Essential oil | Leaves            | $\delta$ -3-Carene<br><i>p</i> -Cymene                                                    | 61.51<br>14.67                   | [33]       |
| Essential oil | Aerial Parts      | Ascaridole<br><i>Trans</i> -Ascaridole glycol<br><i>p</i> -Cymene                         | 31.20<br>5.60<br>36.30           | [34]       |
| Essential oil | Aerial Parts      | Isoascaridole<br>$\alpha$ -Terpinene<br>2,3-Dehydro-1,4-cineole                           | 15.30<br>15.20<br>55.00          | [35]       |
| Essential oil | Aerial Parts      | Isoascaridole<br><i>Trans</i> -Ascaridole glycol<br><i>p</i> -Cymene                      | 6.33<br>10.07<br>22.40           | [36]       |

Table 1. Cont.

| Extracts      | Part of the Plant | Compound                | Concentration (%) | References |
|---------------|-------------------|-------------------------|-------------------|------------|
| Essential oil | Aerial Parts      | <i>o</i> -Cymene        | 39.20             | [19]       |
|               |                   | $\alpha$ -Terpinene     | 36.80             |            |
|               |                   | Nona-3,5-dien-2-ol      | 10.00             |            |
| Essential oil | Aerial Parts      | <i>p</i> -Cymene        | 19.30             | [37]       |
|               |                   | $\alpha$ -Terpinene     | 13.20             |            |
|               |                   | <i>cis</i> -Ascaridole  | 38.10             |            |
| Essential oil | Aerial Parts      | ( <i>Z</i> )-Ascaridole | 87                | [38]       |
|               |                   | ( <i>E</i> )-Ascaridole | 5.04              |            |
|               |                   | <i>p</i> -Cymene        | 4.83              |            |
| Essential oil | Aerial Parts      | $\alpha$ -Terpinene     | 23.77             | [39]       |
|               |                   | Ascaridole              | 14.48             |            |
|               |                   | <i>p</i> -Cymene        | 12.22             |            |
| Essential oil | Aerial Parts      | <i>p</i> -Cymene        | 14.70             | [40]       |
|               |                   | $\delta$ -3-Carene      | 61.50             |            |
| Essential oil | Aerial Parts      | Ascaridole              | 16.30             | [41]       |
|               |                   | Isoascaridole           | 51.00             |            |
|               |                   | <i>p</i> -Cymene        | 6.70              |            |
| Essential oil | Aerial Parts      | <i>o</i> -Cymene        | 41.46             | [14]       |
|               |                   | (+)-4-Carene            | 56.59             |            |
| Essential oil | Aerial Parts      | Ascaridole              | 60.33             | [42]       |
|               |                   | <i>m</i> -Cymene        | 22.17             |            |
| Essential oil | Whole Plant       | <i>p</i> -Cymene        | 49.60             | [43]       |
|               |                   | $\alpha$ -Terpinene     | 26.81             |            |
| Essential oil | Whole Plant       | Ascaridole              | 87.30             | [44]       |
|               |                   | Isoascaridole           | 8.40              |            |
|               |                   | <i>p</i> -Cymene        | 3.30              |            |
| Essential oil | Aerial Parts      | $\alpha$ -Terpinene     | 37.17             | [45]       |
|               |                   | Isoascaridole           | 20.48             |            |
|               |                   | Ascaridole              | 14.83             |            |

Hsu et al. [23] analyzed the oil obtained from the leaves of *D. ambrosioides* cultivated in Taiwan, and it was found to consist of  $\alpha$ -terpinene (30.5%), *p*-cymene (17.3%), carvacrol (16.2%), and ascaridole (15.1%). In a similar study, Maldaner et al. [22] carried out a study to analyze the chemical composition of essential oil obtained by hydrodistillation of young leaves of *D. ambrosioides* from the Amazon region in Brazil. These authors reported that the main components were oxygenated monoterpenes such as ascaridole, ascaridole glycol, linalool acetate, and dihydrocitronellol acetate, with values of 5.75, 10.58, 11.26, and 19.53%. Similarly, Ez-Zriouli et al. [29] reported that the essential oils obtained from aerial parts of *D. ambrosioides* cultivated in the Region of Safi in Morocco were very rich in monoterpene peroxides and monoterpenes, including  $\alpha$ -terpinene (53.4%), ascaridole (17.7%), and *p*-cymene (12.1%). In the same way, Azghar et al. [30] studied the composition of essential oils obtained by hydrodistillation of the aerial part of *D. ambrosioides* collected in the Region of Eastern Morocco, and they reported that *p*-cymene (31.72%), 4-carene (27.34%), and  $\alpha$ -cyclogeraniol acetate (16.90%) were the main components.

As mentioned above, the ethanolic or methanolic extracts obtained from several parts of the plant have also been analyzed to determine the polyphenolic profile. There-

fore, the analysis by HPLC-DAD revealed the presence of rutin in the crude extract (12.5 mg/g), ethyl acetate (16.5 mg/g), and *n*-butanol (8.85 mg/g), whereas quercetin and chrysin were quantified in chloroform fraction (1.95 and 1.04 mg/g), respectively [49]. Kandsi et al. [1] carried out research to analyze the polyphenolic profile of *D. ambrosioides* flower hydroethanolic extract. These authors reported that the hydroethanolic extract contains mainly syringic acid, quercetin, hesperetin, and luteolin. In a similar study, Li et al. [50] analyzed the polyphenolic profile of ethanolic extract obtained from the aerial part of *D. ambrosioides* using an ultrasonic-assisted extractor. They found that the main components were kaempferitin, kaempferol-3-*O*-apigenin-7-*O*-rhamnoside, and kaempferol-3-*O*-acetylapiogenin-7-*O*-rhamnoside, with a concentration of 33.5, 60.77 and 5.73%, respectively. More recently, Figueroa-Merma et al. [51] analyzed the polyphenolic profile of an extract obtained from aerial parts of *D. ambrosioides* plants grown in Lima. They found that the main components were Kaempferol rhamnosyl-dipentoside and Kaempferol dirhamnoside-hexoside, with values of 160.00 and 155.59 mg/100 g dry weight. Another group of bioactive compounds present in the extracts obtained from aerial parts of *D. ambrosioides* is alkaloids, which are found in high concentrations in this plant, as reported by various authors [49,52]. In this sense, Shah et al. [53] stated that *D. ambrosioides* is rich in 1-Piperoylpiperidine. Another study carried out by Kandsi et al. [52] revealed that the hydroethanolic extracts obtained from the flowers of *D. ambrosioides* are a rich source of alkaloids, such as trisphaeridine, galanthamine, crinine, demethylmaritidine, anhydrolycorine, nor-galanthamine, N-formylnorgalanthamine, peramine, and ergovaline. Figueroa-Merma [51] reported that  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols were found in amounts of 28.78, 7.19, 7.96, and 3.27  $\mu\text{g/g}$  in extracts obtained from leaves of *D. ambrosioides* cultivated in Peru. Previously, Shah and Kanh [53] mentioned that the main phytosterols found in methanolic extracts of *D. ambrosioides* were stigmasterol and  $\beta$ -sitosterol. Coumarins were also present in the extracts obtained from the aerial part of *D. ambrosioides*; thus, Ghareeb et al. [54] reported the presence of 1,2-benzopyrone in the extracts obtained from the leaves of *D. ambrosioides*, while Shah and Khan [53] found that scopoletin is present in methanolic extracts obtained from leaves.

#### 4.2. Antimicrobial Properties of *Dysphania ambrosioides*

The extracts and essential oils obtained from several parts of *Dysphania ambrosioides* have been shown to exhibit antimicrobial activities against various microorganisms, including bacteria, yeasts, and fungi, as shown in Table 2 [41,49,55–60]. These activities make them potential natural preservatives for food and pharmaceutical products, as well as agents for the treatment of microbial infections [29]. In reference to essential oils, De Andrade Santiago et al. [61] assessed the antibacterial properties of essential oil extracted from leaves of *D. ambrosioides*, whose principal components were  $\alpha$ -terpinene and *r*-cymene, against several bacteria strains, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella choleraesuis*. These authors reported minimum inhibitory concentration (MIC) values of 62.5, 250, 125, and 125  $\mu\text{g/mL}$ , respectively. Almeida Bezerra et al. [28] reported that the essential oils that had a high content of  $\alpha$ -terpinene and ascaridole, obtained from leaves of *D. ambrosioides* cultivated in Brazil, demonstrated significant MIC values against *S. aureus* (256  $\mu\text{g/mL}$ ), moderate values against *Pseudomonas aeruginosa* (512  $\mu\text{g/mL}$ ) and low values against *E. coli* (1024  $\mu\text{g/mL}$ ). Kandsi et al. [32] carried out a study to analyze the antibacterial activity against *E. coli*, *S. aureus*, and *Enterococcus faecalis* of essential oil obtained from the stem and flowers of *D. ambrosioides* cultivated in Morocco. These authors reported that the MIC values of essential oil obtained from the stem were 18, 18, and >110  $\mu\text{g/mL}$  for *E. coli*, *S. aureus*, and *E. faecalis*, respectively, while for the essential oil obtained for the flowers, the MIC values

were 6,12, and 105 µg/mL for *E. coli*, *S. aureus*, and *E. faecalis*, respectively. This antibacterial activity could be due to the high content of 4-carene and  $\alpha$ -cyclogeraniol acetate present in essential oil. In a similar study, Azghar et al. [30] studied the effect of essential oil (rich in *p*-cymene and 4-carene) obtained from the aerial part of *D. ambrosioides* on multidrug-resistant *E. coli*, *Acinetobacter baumannii*, *P. aeruginosa*, and methicillin-resistant *S. aureus*, reporting MIC values of 150,120, 140 and 230 µg/mL, respectively.

**Table 2.** Antibacterial properties of essential oils and extracts obtained from different parts of *Dysphania ambrosioides*.

| Extracts                                  | Part of the Plant | Main Compound                                             | Microorganism                   | MIC (µg/mL) | Ref. |
|-------------------------------------------|-------------------|-----------------------------------------------------------|---------------------------------|-------------|------|
| Essential oil                             | Leaves            | <i>o</i> -Cymene<br>$\alpha$ -Terpinene                   | <i>Escherichia coli</i>         | 10          | [5]  |
|                                           |                   |                                                           | <i>Staphylococcus aureus</i>    | 10          |      |
|                                           |                   |                                                           | <i>Pseudomonas aeruginosa</i>   | 20          |      |
|                                           |                   |                                                           | <i>Bacillus subtilis</i>        | 20          |      |
| Essential oil                             | Aerial Parts      | 4-Carene<br><i>Trans</i> - $\beta$ -Terpinyl<br>butanoate | <i>E. coli</i>                  | 6           | [32] |
|                                           |                   |                                                           | <i>S. aureus</i>                | 12          |      |
|                                           |                   |                                                           | <i>Enterococcus faecalis</i>    | 18          |      |
| Essential oil                             | Leaves            | $\beta$ -Cymene                                           | <i>S. aureus</i>                | 10          | [25] |
|                                           |                   |                                                           | <i>P. aeruginosa</i>            | 10          |      |
| Essential oil                             | Aerial Parts      | <i>o</i> -Cymene<br>$\alpha$ -Terpinene                   | <i>E. coli</i>                  | 7.8         | [37] |
|                                           |                   |                                                           | <i>B. subtilis</i>              | 3.9         |      |
| Essential oil                             | Leaves            | $\alpha$ -Terpinene<br>Ascaridole                         | <i>E. coli</i>                  | 1024        | [28] |
|                                           |                   |                                                           | <i>S. aureus</i>                | 256         |      |
|                                           |                   |                                                           | <i>P. aeruginosa</i>            | 512         |      |
| Essential oil                             | Aerial Parts      | $\alpha$ -Terpinene<br>Ascaridole                         | <i>E. coli</i>                  | 310         | [40] |
|                                           |                   |                                                           | <i>S. aureus</i>                | 1250        |      |
|                                           |                   |                                                           | <i>P. aeruginosa</i>            | 10,000      |      |
|                                           |                   |                                                           | <i>Klebsiella pneumoniae</i>    | 20,000      |      |
| Essential oil                             | Aerial Parts      | $\delta$ -3-Carene<br><i>p</i> -Cymene                    | <i>Microcystis aeruginosa</i>   | 3120        | [41] |
| Essential oil                             | Aerial Parts      | <i>p</i> -Cymene<br>4-Carene                              | <i>E. coli</i>                  | 90          | [30] |
|                                           |                   |                                                           | <i>S. aureus</i>                | 120         |      |
|                                           |                   |                                                           | <i>P. aeruginosa</i>            | 120         |      |
| Essential oil                             | Leaves            | No Determined                                             | <i>S. aureus</i>                | 1024        | [55] |
| Essential oil                             | Leaves            | $\alpha$ -Terpinene                                       | <i>S. aureus</i>                | 1024        | [56] |
| Essential oil                             | Aerial Parts      | <i>cis</i> -Ascaridole<br><i>m</i> -Cymene                | <i>P. aeruginosa</i>            | 19          | [43] |
|                                           |                   |                                                           | <i>Bacillus subtilis</i>        | 19          |      |
| Ethanollic extract                        | Stem              | Rutin<br>Quercetin                                        | <i>B. subtilis</i>              | 11.1        | [12] |
| Ethanollic extract                        | Leaves            | No determined                                             | <i>E. coli</i>                  | 25,000      | [57] |
|                                           |                   |                                                           | <i>S. aureus</i>                | 25,000      |      |
| Ethanollic extract                        | Leaves            | No determined                                             | <i>Clostridioides difficile</i> | 3900        | [58] |
| Chloroform extract                        | Leaves            | Quercetin<br>Chrysin                                      | <i>S. aureus</i>                | 4290        | [49] |
|                                           |                   |                                                           | <i>Enterococcus faecalis</i>    | 4290        |      |
| Ethanollic extract                        | Aeria Parts       | No determined                                             | <i>E. coli</i>                  | 1094        | [59] |
|                                           |                   |                                                           | <i>E. faecalis</i>              | 4375        |      |
| Ethanollic<br>and methanollic<br>extracts | Aeria Parts       | No determined                                             | <i>E. coli</i>                  | 9           | [60] |
|                                           |                   |                                                           | <i>S. aureus</i>                | 9           |      |
|                                           |                   |                                                           | <i>P. aeruginosa</i>            | 43          |      |
|                                           |                   |                                                           | <i>B. subtilis</i>              | 9           |      |

MIC: minimum inhibitory concentration.

The extracts (ethanollic, methanollic, aqueous, and n-hexane) obtained from the different parts of the *D. ambrosioides* plant also have demonstrated antibacterial activity against several Gram+ and Gram− strains. In this context, Knauth et al. [59] reported that the



methanolic extract from the fruit of *D. ambrosioides* cultivated in Mexico, rich in flavonoids, inhibited the bacterium *Enterococcus faecalis*, *E. coli*, and *Salmonella typhimurium*, with MIC values of 4375, 1094, and 137 µg/mL, respectively. More recently, Ouadja et al. [62] analyzed the antibacterial activity of the ethanolic extract from the leaves of *D. ambrosioides* cultivated in Togo, with a high content of phytol. They reported MIC values of 500 mg/mL against *S. aureus*, *P. aeruginosa*, and *Citrobacter freundii*. Similarly, Bano et al. [63] reported that the n-hexane extracts (100 µg/disc) obtained from *D. ambrosioides* seeds cultivated in Pakistan had high antibacterial activity against *Klebsiella pneumonia*, *Micrococcus luteus*, and *S. aureus*, with inhibition halos of 14, 13, and 9 mm, respectively. This activity probably could be due to the high content of quercetin and kaempferol found in the extracts. Martínez-Alva et al. [58] reported the use of leaf extracts from *D. ambrosioides* cultivated in Mexico City, rich in alkaloids, which showed antibacterial activity against *Clostridioides difficile*, with a MIC value of 3900 µg/mL. The antibacterial activity of *Dysphania* extract and essential oil makes it a promising natural alternative to synthetic bactericides. The oil could be used as a disinfectant in healthcare settings, reducing the risk of hospital-acquired infections [55]. It could also be used in food preservation, extending the shelf life of perishable products and reducing the risk of foodborne illnesses [59]. Additionally, the extracts could be used in pharmaceutical applications, such as in the development of novel antibacterial agents. The antibacterial activity of *Dysphania* is attributed to the synergistic effects among several compounds. Terpenes, such as α-terpinene and o-cymene, are known to disrupt the bacterial cell membrane, leading to leakage of cellular contents and ultimately, cell death [64]. In addition, as mentioned by Musa et al. [65] and Singh and Pandey [2], the bioactive compounds present in *D. ambrosioides* are mainly hydrophobic, which allows them to enter the bacterial cell membrane and mitochondria, disrupt the cellular structure, and produce the death of bacteria. In addition to antibacterial activity, extracts or essential oils obtained from *D. ambrosioides* have also been shown to possess significant antifungal activity. Therefore, in the scientific literature, it is possible to find several studies where the antifungal activity of *D. ambrosioides* extracts or essential oil has been determined. Stappen et al. [34] assessed the antifungal activity of the essential oil obtained from leaves and inflorescence of *D. ambrosioides* cultivated in western Himalaya against *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, and *Colletotrichum fragariae*. They found that the inhibition zones of fungal growth were between 6.5 and 8.0 mm when concentrations of 80 µg/spot were used, and inhibition zones of 11.0 to 14.5 mm were observed for concentrations of 160 µg/spot. Mokni et al. [43] evaluated the antifungal activity of the essential oil obtained from fresh leaves of *D. ambrosioides* collected in northwestern Tunisia. These authors informed that this essential oil showed antifungal activity against the pathogenic strain *Candida albicans* yeast, with MIC values equal to 39 µg/mL. In a similar study, Almeida Bezerra et al. [28] evaluated the cellular viability of different strains of *C. albicans* and *Candida tropicalis* when exposed to essential oil obtained from the leaves of *D. ambrosioides* cultivated in Brazil. It is possible to notice that, for *C. albicans* LM77, the essential oil had an IC<sub>50</sub> value of 19.3 µg/mL, while for *C. albicans* INCQS 40006, the IC<sub>50</sub> value was 25.2 µg/mL. Regarding the cellular viability of *C. tropicalis* LM 23, the essential oil showed antifungal activity, with an IC<sub>50</sub> of 101.9 µg/mL and an IC<sub>50</sub> of 15.8 µg/mL for the *C. tropicalis* INCQS 40042 strains. Zefzoufi et al. [21] found that the essential oil obtained from leaves at µg/mL inhibited the growth of *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tabaci*, and *Erwinia amylovora*. More recently, Hsu et al. [23] carried out a study to analyze the antifungal activity against wood decay fungi of essential oils obtained from fresh leaves of *D. ambrosioides* cultivated in Taiwan. They reported a complete inhibition of the fungal *Phaeolus schweinitzii* and *Lenzites sulphureus*, with a concentration of essential oil of 50 µg/mL, while for *Phaneochaete*

*chrysosporium*, the concentration required was 100 µg/mL. Finally, for *Trametes versicolor*, 200 µg/mL of essential oil was necessary for inhibition. In reference to the MIC values, these authors reported values of 0.10, 0.10, 0.10, and 0.05 mg/mL for *T. versicolor*, *P. schweinitzii*, *P. chrysosporium*, and *L. sulphureus*, respectively.

The antifungal properties of extracts obtained from *D. ambrosioides* have also been determined. In this sense, Bano et al. [63] reported that chloroform and acetone–methanol *D. ambrosioides* seed extracts had great activity against *Fusarium solani* and *Aspergillus fumigatus*, with inhibition zones of 17 and 12 mm, respectively. These authors reported MIC values of 100 µg/disc against *Aspergillus niger*, *A. fumigatus*, *Aspergillus flavus*, *Fusarium solani*, and *Mucor* spp. Similarly, Gishen et al. [57] conducted a study to analyze the antifungal activity against *C. albica* of ethanolic extracts obtained from fresh leaves of *D. ambrosioides* cultivated in Ethiopia. They found that the analyzed extract showed a minimum inhibition zone concentration of 1 g/mL. The high antifungal activity of *D. ambrosioides* might be due to the essential oil and extracts having a high amount of ascaridole in their composition. This is a bicyclic monoterpene with a rather unusual bridging peroxide functional group and has long been the only known natural peroxide [34]. Moreover, besides the main ingredients, minority components can also play an important role in the antifungal activity of extracts. Multiple botanical chemicals and essential oils present in plants may exert synergistic or antagonistic effects [63]. Phenolic compounds might be responsible for antifungal activity. From our perspective, and based on the results reported in the analyzed papers, *D. ambrosioides* essential oil is not the most effective plant-derived essential oil for controlling bacteria and fungi. However, it is a viable alternative for extending the shelf life of various foods, due to its ability to reduce microorganism growth. It should be noted that its activity is reduced when applied to several matrices. On the other hand, given its properties, we believe its use would be valuable in barrier technology for food preservation.

#### 4.3. Antioxidant Properties of *Dysphania ambrosioides*

*Dysphania ambrosioides* extracts and essential oils have been found to possess antioxidant and anti-inflammatory properties, which can help protect against oxidative stress and inflammation-related diseases. These activities make them potential natural remedies for the prevention and treatment of chronic diseases such as cancer, cardiovascular disease, and neurodegenerative disorders [7,66].

There exists a marked interest in natural antioxidants, particularly in compounds such as flavonoids and other polyphenols, including tannins, abundantly present in plants like *D. ambrosioides*. In the scientific literature, it was possible to find several studies where the antioxidant activity of *D. ambrosioides* extracts or essential oil obtained from different parts of the plant, including the aerial part, flowers, seeds, stems, and leaves, had been determined, as shown in Table 3 [60,63,66–69].

**Table 3.** Antioxidant properties of essential oils and extracts obtained from different parts of *Dysphania ambrosioides*.

| Extracts              | Part of the Plant Used | Main Component                           | Methodology         | Quantity                                                                      | Reference |
|-----------------------|------------------------|------------------------------------------|---------------------|-------------------------------------------------------------------------------|-----------|
| Ethyl Acetate extract | Aerial Part            | No determined                            | FRAP<br>ABTS        | 12.90 mg/mL<br>4.56 mg/mL                                                     | [66]      |
| Aqueous extract       | Leaves                 | 16-methyl-heptadecane-1,2-diol<br>Phytol | FIC<br>FRAP<br>DPPH | IC <sub>50</sub> 20.98 mg/mL<br>64.19 mg/AAG g<br>IC <sub>50</sub> 1.39 mg/mL | [11]      |

Table 3. Cont.

| Extracts                                 | Part of the Plant Used | Main Component                                 | Methodology                              | Quantity                                                                                       | Reference |
|------------------------------------------|------------------------|------------------------------------------------|------------------------------------------|------------------------------------------------------------------------------------------------|-----------|
| Methanolic extract                       | Seeds                  | Rutin                                          | DPPH<br>ABTS<br>FRAP                     | IC <sub>50</sub> 110.7 µg/mL<br>110.6 µg AAE/mg<br>94.30 µg AAE/mg                             | [63]      |
| Aqueous extract                          | Fruits                 | No determined                                  | ABTS<br>FIC                              | 8.25 mM TE/g<br>78% quelation                                                                  | [60]      |
| <i>n</i> -Butanol ethyl acetate extracts | Leaves                 | Caffeic acid<br>Coumarin<br>Kaempferol         | DPPH                                     | IC <sub>50</sub> 2.98 mg/mL<br>IC <sub>50</sub> 16.48 mg/mL                                    | [54]      |
| Methanolic extract                       | Leaves                 | Rutin<br>Quercetin                             | DPPH                                     | IC <sub>50</sub> 130.7 µg/mL                                                                   | [12]      |
| Methanolic extract                       | Leaves                 | No determined                                  | FRAP<br>ABTS                             | 0.141 µM TE/g<br>0.224 mg AAE/g                                                                | [67]      |
| Hydroethanolic extract                   | Flowers                | Syringic acid<br>Quercetin<br>Kaempferol       | DPPH<br>β-Carotene/linoleic acid<br>FRAP | IC <sub>50</sub> 166.47 µg/mL<br>IC <sub>50</sub> 57.04 µg/mL<br>IC <sub>50</sub> 231.05 µg/mL | [1]       |
| Aqueous extract                          | Aerial Part            | No determined                                  | DPPH<br>ORAC                             | IC <sub>50</sub> 80.6 µg TE/mL<br>IC <sub>50</sub> 687.3 µg TE/mL                              | [68]      |
| Essential oil                            | Aerial Part            | α-Terpinene<br>Ascaridole                      | DPPH                                     | 30.182 mg TE/g oil                                                                             | [29]      |
| Essential oil                            | Leaves                 | No determined                                  | DPPH<br>FRAP<br>ABTS                     | 1.59 mg AAE/g<br>8.36 mg AAE/g<br>2.11 mg AAE/g                                                | [59]      |
| Essential oil                            | Stems                  | 4-Carene<br>α-Cyclogeraniol<br>Acetate         | FRAP<br>ABTS                             | IC <sub>50</sub> 309.45 µg/mL<br>IC <sub>50</sub> 147.99 µg/mL                                 | [32]      |
|                                          | Flower                 | <i>trans</i> -β-TerpinyolButanoate<br>4-Carene | DPPH<br>β-Carotene/linoleic acid         | IC <sub>50</sub> 158.15 µg/mL<br>IC <sub>50</sub> 266.25 µg/mL                                 |           |
| Essential oil                            | Leaves                 | α-Terpinene<br>Ascaridole                      | DPPH                                     | IC <sub>50</sub> 1024 µg/mL                                                                    | [28]      |
| Essential oil                            | Aerial Part            | α-Terpinene<br>Ascaridole                      | DPPH<br>β-Carotene/linoleic acid<br>FRAP | IC <sub>50</sub> 4.00 mg/mL<br>IC <sub>50</sub> 3.03 µg/mL<br>IC <sub>50</sub> 6.02 µg/mL      | [40]      |
| Essential oil                            | Leaves                 | α-Terpinene<br>α-Terpinenyl Acetate            | DPPH                                     | IC <sub>50</sub> 1.74 mg/mL                                                                    | [69]      |

FRAP: ferric reducing power assay; ABTS: (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) method; DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; FIC: ferrous ion chelating assay; TE: Trolox equivalent. IC<sub>50</sub>: its ability to eliminate 50% of free radicals; AAE: ascorbic acid equivalents.

De Andrade Santiago et al. [61] assessed the antioxidant capacity of essential oil extracted from leaves of *D. ambrosioides*, whose principal components were α-terpinene and *r*-cymene, using two different methodologies such as the β-carotene–linoleic acid and DPPH assays. These authors reported an IC<sub>50</sub> value in the β-carotene–linoleic acid test of

455.7 µg/mL, while for the DPPH assay, 500 µg/mL resulted in the inhibition of radical DPPH by 15.79%. Villalobos-Delgado et al. [70] studied the antioxidant activity of ethanolic extract (1 g/20 mL) obtained from leaves of *D. ambrosioides* cultivated in Mexico and reported the inhibition of the radical measured with the DPPH assay by 16.65%. This antioxidant activity could be due to the high content of quercetin and kaempferol *o*-rhamnosyl-pentoside present in the extract. Ogunleye et al. [11] conducted a study to analyze the antioxidant properties of extracts obtained from the aerial parts of *D. ambrosioides* cultivated in Nigeria, which showed a high concentration of 16-methyl-heptadecane-1,2-diol and phytol. The antioxidant assays revealed that *D. ambrosioides* leaf extracts possess antioxidant properties, with hexane fraction exhibiting the highest scavenging activities for DPPH, with an IC<sub>50</sub> value of 0.02 mg/mL, while in the FRAP assay, the value obtained was 730.92 mg ascorbic acid equivalent/g. Ouadja et al. [62] determined the antioxidant capacity of hydroethanolic extract, with a high content of phytol obtained from the leaves of *D. ambrosioides* cultivated in Togo using three different methodologies, namely FRAP and ABTS assays. They reported antioxidant activity values of 32.48 and 45.33 µg ascorbic acid equivalent/mg for samples subjected to the FRAP and ABTS assays, respectively. In this sense, Pandiangan et al. [71] analyzed the antioxidant activity (DPPH assay) of water and acetone extracts obtained from the leaves of *D. ambrosioides* cultivated in Indonesia. They reported that the IC<sub>50</sub> value of the acetone extract of *D. ambrosioides* was 9.7 µg/mL, while the IC<sub>50</sub> value of the water extract was 1.32 µg/mL. Tchani et al. [72] analyzed the antioxidant activity of ethanolic and aqueous extracts, which showed a high concentration of flavonoids, obtained from the leaves and seeds of *D. ambrosioides* by maceration and infusion. These authors revealed that infusion yielded IC<sub>50</sub> values of 25.541 and 48.269 µg/mL in aqueous and ethanolic media, respectively, whereas maceration resulted in IC<sub>50</sub> values of 29.18 and 50.99 µg/mL, respectively. In a similar study, Kandsi et al. [32] analyzed the antioxidant properties of essential oils obtained from *D. ambrosioides* leaves cultivated in Morocco at concentrations ranging between 25 and 400 µg/mL using the DPPH and β-carotene assays. They reported IC<sub>50</sub> values of 210.24 and 220.50 µg/mL for DPPH and β-carotene assays, respectively. This antioxidant activity could be due to the content of 4-carene and α-cyclogeraniol acetate. In this way, Bano et al. [63] reported that the antioxidant capacity of methanolic extracts, which showed a high content of quercetin and kaempferol, obtained from *D. ambrosioides* seeds cultivated in Pakistan and measured with ABTS and FRAP assays, was 110.6 and 94.3 µg ascorbic acid equivalents/mg extract, respectively, while the *n*-hexane extract revealed the lowest antioxidant potential, with values of 11.2 and 13.7 ascorbic acid equivalents/mg extract for ABTS and FRAP assays, respectively. Ez-Zriouli et al. [29] investigated the antioxidant activity of essential oil rich in ascaridole and α-terpinene, which was obtained from the aerial part *D. ambrosioides* collected in Morocco, and they reported values of 30.82 mg Trolox equivalent per g of essential oil. More recently, Drioua et al. [66] assessed the antioxidant activity of ethyl acetate fraction from the aerial components of *D. ambrosioides* cultivated in Morocco, which showed a high content of flavonoids and phenolic acids, employing the DPPH assay. They reported that these extracts yielded an IC<sub>50</sub> value of 0.54 mg/mL.

#### 4.4. Insecticidal and Repellent Activities of *Dysphania ambrosioides*

The extracts and essential oils obtained from several plants have been used for insect control for centuries around the world and are considered safe due to their relatively short shelf life and low toxicity to humans and animals [73]. In this way, the extracts and essential oils of *D. ambrosioides* have been shown to possess insecticidal and repellent activities against mosquitoes and other insects, making them potential natural insecticides

and repellents for the control of vector-borne diseases [34], as essential oils negatively affect the feeding, growth, reproduction, and oviposition of harmful insects [73]. In the scientific literature, there were several studies [34,37,38,73–80] where the insecticidal and repellent activities of *D. ambrosioides* extracts or essential oil had been determined (Table 4).

**Table 4.** Insecticidal and repellent activities of essential oils and extracts obtained from different parts of *Dysphania ambrosioides*.

| Extracts      | Part of the Plant Used | Main Compounds                             | Quantity                                     | Insects                                          | Reference |
|---------------|------------------------|--------------------------------------------|----------------------------------------------|--------------------------------------------------|-----------|
| Essential oil | Leaves                 | No determined                              | 1 µL/L air                                   | <i>Periplaneta americana</i>                     | [73]      |
| Essential oil | Aerial Parts           | Terpinolene<br><i>p</i> -Cymene            | 1.02 µL/L air                                | <i>Dactylopius opuntiae</i>                      | [74]      |
| Essential oil | Aerial Parts           | <i>cis</i> -Ascaridole<br><i>p</i> -Cymene | 3.125 µL/L                                   | <i>Culex quinquefasciatus</i>                    | [38]      |
| Essential oil | Aerial Parts           | <i>p</i> -Cymene<br>Ascaridole             | 62.5 mg/L<br>10 mg/L                         | <i>Aedes aegypti</i><br><i>Anopheles gambiae</i> | [34]      |
| Essential oil | Aerial Parts           | <i>o</i> -Cymene<br>α-Terpinene            | 0.75 mg/mL                                   | <i>Culex pipiens</i>                             | [37]      |
| Essential oil | Whole Plant            | No determined                              | 66.81 mg/L                                   | <i>Plutella xylostella</i>                       | [44]      |
| Essential oil | Leaves                 | δ-3-Carene<br><i>p</i> -Cymene             | 0.04 µL/cm <sup>2</sup>                      | <i>Tribolium confusum</i>                        | [33]      |
|               | Inflorescences         | δ-3-Carene<br><i>p</i> -Cymene             | 0.05 µL/cm <sup>2</sup>                      |                                                  |           |
| Essential oil | Leaves                 | No determined                              | 0.50 mg/m <sup>2</sup>                       | <i>Callosobruchus maculatus</i>                  | [75]      |
| Essential oil | Fresh Leaves           | Ascaridole<br><i>p</i> -Cymene             | LC <sub>50</sub><br>17.74 µg/cm <sup>2</sup> | <i>Alphitobius diaperinus</i>                    | [76]      |
| Extracts      | Aerial Parts           | No determined                              | 50 g/L                                       | <i>Dactylopius opuntiae</i>                      | [77]      |
| Extracts      | Aerial Parts           | No determined                              | 10 g/L                                       | <i>Bemisia tabaci</i>                            | [78]      |
| Extracts      | Aerial Parts           | No determined                              | 200 g/L                                      | <i>Spodoptera frugiperda</i>                     | [79]      |
| Extract       | Stem and Leaves        | No determined                              | 500 mg/mL                                    | <i>Scyphophorus scupunctatus</i>                 | [80]      |

LC<sub>50</sub>: lethal concentration 50%.

Vite-Vallejo et al. [78] evaluated insecticidal activity against *Bemisia tabaci* using ethanolic extracts obtained from *D. ambrosioides*, cultivated in Mexico. They reported that the extracts of *D. ambrosioides* at concentrations of 1, 2, 3, 4, 5, and 6% killed 3.5, 10, 26, 70, 88, and 93% of *B. tabaci*, respectively. In a similar study, Stappen et al. [34] analyzed the insecticidal activity of essential oils rich in ascaridole and *p*-cymene, obtained from the leaves of *D. ambrosioides* collected from India, against *Aedes aegypti*. These authors mentioned that *D. ambrosioides* essential oil had a mortality of 100%, with a concentration of 125 mg/L, while the mortality at a concentration of 62.5 mg/L was 80%. Langsi et al. [81] reported that the essential oil obtained from leaves of *D. ambrosioides*, which showed a high content in 4-carene and *p*-cymene, caused at least 80% *Sitophilus zeamais* mortality within 14 days of storage with a dose of 200 µL/kg. In addition, 8 µL of essential oil was repellent to the weevils. Velez et al. [77] reported that the extract obtained from the leaf powder of *D. ambrosioides* grown in Brazil showed insecticidal activity against *Dactylopius opuntiae*. They reported that aqueous *D. ambrosioides* extract at 10% had a corrected mortality of 24.41%, while the corrected mortality of hydroethanolic *D. ambrosioides* extract at 5% was



17.49%. Almadiy [38] conducted a study to analyze insecticidal activity against the larvae and adults of *Culex quinquefasciatus* using essential oils obtained by hydrodistillation from aerial parts of *D. ambrosioides* grown in Saudi Arabia, which had a high concentration of (Z)-ascaridole. These authors reported that the larval mortality varied between 16.30 and 30.34% when the lower concentration (3.125  $\mu\text{L/L}$ ) was used, while for the higher tested concentrations (50  $\mu\text{L/L}$ ), the mortality increased to 80.11–100.00% after 24 h of treatment. Finally, they found that all treatments for larval and adult mortality were time- and dose-dependent. More recently, Laghzaoui et al. [74] reported that the essential oils obtained from leaves of *D. ambrosioides* cultivated in Morocco were toxic to the adult males and crawlers of *Dactylopius opuntiae*. The authors reported that this essential oil, which had a high content of isoascaridole and carvacrol, showed an  $\text{LC}_{50}$  and 90% lethal concentration ( $\text{LC}_{90}$ ) of 0.004 and 0.009  $\mu\text{L}/\text{cm}^2$ , respectively, against adult males of *D. opuntiae* using contact bioassay. On the other hand, the values obtained for  $\text{LC}_{50}$  and  $\text{LC}_{90}$  of essential oil against crawlers of *D. opuntiae* using contact bioassay were 0.003 and 0.018  $\mu\text{L}/\text{cm}^2$ , respectively. In a more recent study, Yikinc and Tunaz [73] analyzed the insecticidal activity of essential oils obtained from leaves of *D. ambrosioides* against *Periplaneta americana*. These authors found that higher mortality rates of *P. americana* adults occurred at a concentration of 5  $\mu\text{L/L}$  of the essential oils derived from *D. ambrosioides*, while with a concentration of 2.5  $\mu\text{L/L}$  of *D. ambrosioides* essential oil, after 24 h, 100% mortality was achieved for *P. americana* adults. Kasrati et al. [33] conducted a study to analyze the insecticidal activity of essential oils obtained from the leaves and inflorescences of *D. ambrosioides* against *Tribolium confusum* adults. These authors reported that these essential oils had an  $\text{LD}_{50}$  of 4.30 and 4.46  $\mu\text{L/L}$  air and  $\text{LD}_{90}$  of 6.51 and 9.62  $\mu\text{L/L}$  air for toxicity by fumigation. This activity could be due to the high content of  $\delta$ -3-carene and *p*-cymene found in this essential oil.

#### 4.5. Antiparasitic Activities of *Dysphania ambrosioides*

Annual yield losses in vegetables caused by phytonematodes in the world are estimated at 11% [82]. The use of *D. ambrosioides* extracts in the control of root-knot nematodes is even more important in family farming since horticulturists have low purchasing power and little access to more advanced technologies. Extracts and/or essential oils represent a technically viable and low-cost option compared to the main traditional control techniques [82]. The extracts and essential oils of *D. ambrosioides* yield promising results probably due to the complex mixtures of compounds from plant secondary metabolism that act as antimicrobial, antiviral, and antifeedant agents to protect plants [24].

Essential oils exhibit broad activity against parasitic microorganisms since they negatively affect the feeding, growth, reproduction, and oviposition of harmful parasites [73]. Based on the induction of different mitochondrial targets, many authors have attributed the antiparasitic activity to the major chemical components, namely carvacrol, caryophyllene oxide, and ascaridole [83,84]. The scientific literature presents several studies [42,45,82,85–89] where the antiparasitic activities of *D. ambrosioides* extracts or essential oil have been studied (Table 5).

**Table 5.** Antiparasitic activities of essential oils and extracts obtained from different parts of *Dysphania ambrosioides*.

| Extracts      | Part of the Plant | Main Compound                  | Concentration        | Parasites                    | Reference |
|---------------|-------------------|--------------------------------|----------------------|------------------------------|-----------|
| Essential oil | Fruit and Seeds   | (Z)-Ascaridole<br>E-Ascaridole | 307 $\mu\text{g/mL}$ | <i>Meloidogyne incognita</i> | [45]      |

Table 5. Cont.

| Extracts      | Part of the Plant | Main Compound                                               | Concentration | Parasites                      | Reference |
|---------------|-------------------|-------------------------------------------------------------|---------------|--------------------------------|-----------|
| Essential oil | Aerial Part       | 4-Carene<br>o-Cymene                                        | 4.74 mg/mL    | <i>Leishmania tropica</i>      | [15]      |
| Essential oil | Aerial Part       | <i>cis</i> -Piperitone oxide<br><i>trans</i> -Isoascaridole | 8.7 µg/mL     | <i>Trypanosoma cruzi</i>       | [24]      |
| Essential oil | Aerial Part       | No determined                                               | 50 µL/mL      | <i>Ancylostoma</i> spp.        | [39]      |
| Essential oil | Aerial Part       | <i>p</i> -Cymene<br>α-terpinene                             | 0.037 µL/g    | <i>Rhipicephalus lunulatus</i> | [85]      |
| Essential oil | Flower            | Isoascaridole<br>Ascaridole                                 | 0.041 µL/mL   | <i>Meloidogyne chitwoodi</i>   | [42]      |
| Extracts      | Aerial Part       | No determined                                               | 20 mg/mL      | <i>Haemonchus contortus</i>    | [86]      |
| Extracts      | Aerial Part       | No determined                                               | 0.6 mg/mL     | <i>Haemonchus contortus</i>    | [87]      |
| Extracts      | Aerial Part       | No determined                                               | 400 mg/mL     | <i>Rhipicephalus microplus</i> | [88]      |
| Extracts      | Leaves            | No determined                                               | 300 mg/mL     | <i>Meloidogyne javanica</i>    | [82]      |
| Extracts      | Leaves            | No determined                                               | 50 mg/mL      | <i>Meloidogyne enterolobi</i>  | [89]      |
| Extracts      | Whole Plant       | Rutin<br>Quercetin                                          | 1 mg/mL       | <i>Leishmania tropica</i>      | [12]      |

In a study carried out by Guimarães et al. [82], antiparasitic activity against *Meloidogyne javanica* and *Abelmoschus esculentus* was analyzed using the extracts obtained from the leaves of *D. ambrosioides* collected in Pakistan. The plant extract of *D. ambrosioides* yielded a lower reproduction factor (FR = 11.80) of the nematode in the roots of the *Abelmoschus esculentus*. In addition, *M. javanica* was reduced by 57.51%. In a similar study, Ajaib et al. [86] assessed the anthelmintic activity of the extracts obtained from *D. ambrosioides* leaves collected in Pakistan against *Haemonchus contortus*. Chloroform extract took the minimum time for the paralysis and death of worms, with values of 12 and 17 h at 100 mg/mL concentration, whereas at 20 mg/mL, the maximum time taken for the paralysis and death of worms was also by chloroform extract, with values of 99 and 115 h. Zamilpa et al. [87] assessed the in vitro nematocidal effect of *D. ambrosioides* *n*-hexane cultivated in Mexico against *H. contortus* infective larvae. The results showed that the highest individual lethal in vitro effect (96.3%) was obtained with the *D. ambrosioides* extract at 72 h post-confrontation at 40 mg/mL, while the highest combined effect (98.7%) was obtained after 72 h at 40 mg/mL. The in vivo assay showed that the individual administration of the *D. ambrosioides* extracts reduced the parasitic burden in gerbils by 45.8%. Bernardes et al. [90] found that the essential oil obtained from leaves at a concentration of 20.0 µL/L was capable of killing 100% of adults of *Zabrotes subfasciatus* and demonstrated effective repellent activity at 0.8 µL/L air and Lethal Dose<sub>50</sub>. Niaz et al. [15] conducted a study to analyze anti-leishmanial activity against *Leishmania tropica* promastigotes using the essential oil obtained from fresh aerial parts through steam distillation of *D. ambrosioides* collected from Pakistan, which showed a high concentration of 4-carene and *o*-cymene. These authors reported that the essential oil of *D. ambrosioides* had a very potent anti-leishmanial activity with, an LC<sub>50</sub> of Log<sub>10</sub> 1.83 × 10<sup>−6</sup> mg/mL. The low LC<sub>50</sub> value indicates that the essential oil is

very potent against *Leishmania tropica*. In a similar study, Pagotti et al. [24] explored the in vitro and/or in vivo trypanocidal (Chagas disease) activities of the essential oil obtained from *D. ambrosioides* collected from Brazil. *D. ambrosioides* oil was the most active against the trypomastigote and amastigote forms of *Trypanosoma cruzi* in vitro; the IC<sub>50</sub> values were 8.7 and 12.2 µg/mL, respectively. The authors reported that this essential oil, which showed a high content of cis-piperitone oxide and trans-isoascaridole, had a high selectivity index (SI) for trypomastigote (SI = 33.2) and amastigote (SI = 11.7) forms. On day 7, in vivo treatment with *D. ambrosioides* at 20 mg/kg/day reduced parasitemia by 6.36%. Barros et al. [45] evaluated the essential oil of *D. ambrosioides*, extracted from the aerial parts of *D. ambrosioides* plants grown in Brazil, to determine its ability to inhibit the hatching of *Meloidogyne incognita* in vitro. They found that, at 0.5 mg/mL, the essential oil of *D. ambrosioides*, which showed a high content of α-terpinene and isoascaridole, induced mortality in the juvenile larvae of *Meloidogyne incognita* by more than 90%, with LC<sub>50</sub> and LC<sub>90</sub> values of 0.31 and 0.58 mg/mL, respectively. Soares et al. [91] reported that the essential oil (rich in cis-piperitone oxide and p-cymene) obtained from leaves of *D. ambrosioides* at concentrations of 25 and 12.5 µg/mL exhibited notable schistosomicidal action against *Schistosoma mansoni*. At this concentration, the essential oil killed 100% of adult worm pairs within 24 h. The LC<sub>50</sub> values reported were 6.50, 3.66, and 3.65 µg/mL at 24, 48, and 72 h, respectively.

It is important to highlight that, when comparing the potential of *Dysphania ambrosioides* with other genera or species used for similar applications, it stands out for its anthelmintic effect, primarily due to the presence of ascaridole in its essential oil. Although other plants possess anthelmintic activity, *D. ambrosioides* is particularly noted for its use against root-knot nematodes. In addition, it has some pesticidal and insect-repellent properties (not very common in other genera) that are very valuable for organic farming. In addition, it grows easily in different conditions, thus enabling wide availability for processing at the industrial level. However, its toxicity is a significant drawback compared to other medicinal plants, especially essential oils; thus, its use requires caution and strong control of the doses applied to ensure the safe use of the treatment.

#### 4.6. Other Biological Properties of *Dysphania ambrosioides*

Several studies have demonstrated the biological effects of *D. ambrosioides* essential oil and extracts. In reference to cancer, numerous studies reported that the extracts or components found in *D. ambrosioides* have a protective effect on the development and proliferation of some cancer cell lines. Therefore, Tauchen et al. [68] reported that *D. ambrosioides* extracts had an antiproliferative effect on a wide spectrum of cancer cells, including Caco-2, HT-29, and Hep-G2, with IC<sub>50</sub> values of 29.2, 69.9, and 130.6 µg/mL, respectively. In another study, Shameem [46] analyzed the antiproliferative ability of *D. ambrosioides* essential oils against MCF-7 human mammary carcinoma cells and A549 human lung adenocarcinoma epithelial cells. They revealed that the highest antiproliferative activity was observed at 125 µg/mL in the A549 cell line, whereas the growth of the MCF-7 cell line was inhibited at 31.25 µg/mL. Pandiangan et al. [92] mentioned that the extracts obtained from the leaves of *D. ambrosioides* yielded an IC<sub>50</sub> value of 0.105 µg/mL on P388 leukemia cells. Huang et al. [93] reported that the proliferation of human hepatocellular carcinoma cells SMMC-7721 was significantly inhibited by *D. ambrosioides* seed extracts, with IC<sub>50</sub> values of 0.587 g/L, 0.360 g/L, and 0.361 g/L at 24 h, 36 h, and 48 h, respectively. Another biological activity of essential oils or extracts obtained from *D. ambrosioides* is their anti-inflammatory capacity. In this context, Rios et al. [94] mentioned that the hydroethanolic extract obtained from *D. ambrosioides* at a concentration of 5 mg/kg could inhibit nitric oxide and hydrogen peroxide production

and, consequently, reduce inflammation. Ouadja et al. [62] revealed that both the essential oil and hydroethanolic extract obtained from the fresh leaves of *D. ambrosioides* significantly suppressed lipoxygenase (LOX) activity from 95.14 to 98.11 percent at the 1st, 5th, and 10th min of the kinetics. Mokni et al. [43] reported that the essential oil obtained from the fresh leaves of *D. ambrosioides* had a remarkable antiviral activity against Coxsackie Virus-B4, with an  $IC_{50}$  value of 21.75  $\mu\text{g/mL}$ .

#### 4.7. Safety Margins of *Dysphania ambrosioides* Extracts and Essential Oils

While *D. ambrosioides* has a long history of traditional use, improper dosing or concentrated forms can be toxic. The distinction between traditional preparations (like infusions) and concentrated extracts or essential oils is vital.

In reference to the toxicity of *D. ambrosioides* crude extracts, Kandsi et al. [52] reported that the hydroethanolic extracts of *D. ambrosioides* flowers have an oral  $LD_{50}$  (lethal dose 50%, the dose at which 50% of the tested animals die) of 5 g/kg in acute toxicity studies in rats, while the aqueous and methanolic extracts obtained from leaves of *D. ambrosioides* yielded an  $LD_{50}$  greater than 2 g/kg also in rats. Similarly, Drioua et al. [95] revealed that the  $LD_{50}$  of crude extracts obtained from leaves of *D. ambrosioides* was greater than 2 mg/kg.

On the other hand, essential oil is generally considered more toxic than crude extracts due to its concentrated nature and higher content of active compounds, particularly ascaridole. A study carried out by Ez-Zriouli et al. [29] reported an  $LD_{50}$  of 0.5 mg/kg body weight for essential oil in rats, classifying it as a category four cytotoxic natural product at high doses. Adinci Kossi et al. [96] reported that an oral dose of *D. ambrosioides* essential oil at a concentration of 2 g/kg body weight induced death in rats within 24 h.

## 5. Conclusions and Perspectives

The use of artificial intelligence based on natural language processing (NLP), as applied in this study for literature review, represents an innovative tool to enhance the efficiency of scientific analyses. This approach warrants further exploration in future phytochemical and biomedical research. Using this methodology, it was possible to efficiently review and assess the relevance of 814 scientific published in just a few minutes, with a significant improvement over the time required for traditional search processes. Therefore, the usefulness of such a methodology is indisputable, marking a significant change in how state-of-the-art reviews are conducted across any scientific field. In this sense, future studies—regardless of their scientific field or purpose (e.g., projects, review papers, grant applications)—can leverage this tool to reduce selection biases and increase transparency. However, human oversight remains essential, especially in the final phase of the process, where the interpretation and in-depth analysis of selected information are critical. Additionally, this new tool is free to use and available to the entire scientific community, aligning with current open science principles adopted by many regulatory and funding entities, such as the Coalition for Advancing Research Assessment (CoARA) and the National Strategy for Open Science—Spain, which promote transparency in scientific research.

*Dysphania ambrosioides* has demonstrated significant ethnopharmacological potential due to its richness in bioactive compounds, particularly ascaridole, *p*-cymene, and  $\alpha$ -terpinene. The essential oils and extracts of this plant exhibit strong antimicrobial activity against a variety of bacteria and fungi, including multidrug-resistant strains, highlighting its potential as a natural alternative to synthetic antibiotics. Additionally, its antioxidant properties, attributed to its high flavonoid and phenolic content, suggest potential applications against oxidative stress-related diseases. The plant also displays potent antiparasitic

activity, particularly against nematodes and protozoa, supporting its traditional use in treating parasitic infections. Furthermore, its insecticidal and repellent effects position it as a promising candidate for eco-friendly pest control. Despite these promising findings, further research is needed to elucidate the precise mechanisms of action of its active compounds and to assess its safety and efficacy in clinical and agricultural applications. The standardization of extraction methods and toxicity studies are crucial for its integration into pharmaceutical and agricultural industries.

Future research on this topic should focus on the development and optimization of eco-friendly and efficient extraction methods, as well as the isolation and characterization of specific bioactive compounds. These compounds should then be used to elucidate precise mechanisms of action—even investigating underexplored therapeutic potentials—through advanced pharmacological studies. It is also crucial to conduct rigorous toxicological assessments to establish safe therapeutic windows and administration routes. Lastly, large-scale evaluations for specific applications should be performed. Successful execution of these research directions holds the potential not only to scientifically validate and optimize existing traditional uses but also to uncover entirely novel pharmaceutical applications. This could lead to the discovery of new drug candidates derived from natural sources, thus significantly contributing to addressing unmet medical needs and diversifying the global pharmacological arsenal.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants14131903/s1>, Figure S1: Quantitative distribution of the papers in each of the 19 clusters (numbered from 0 to 18); Figure S2. Word clouds of the 75 most common words in each cluster (the number of the cluster is indicated at the top of each word cloud); Figure S3. Frequency bar chart of the 15 most common (the highest frequency appearance) words in each of the 19 clusters (numbered from 0 to 18). Figure bars for the 15 top words (the highest frequency appearance) for each cluster (19 clusters numbered from 0 to 18 and identified at the top of each figure); Figure S4. Scatter plot of the principal component analysis results for the 814 papers included in the datasheet of the initial bibliographic research. Each cluster is represented by a different color, corresponding the black points to the centroids of each cluster; Figure S5. Distribution of the 19 clusters over time (2000–2024). Table S1: Data, IA-based tools, and results obtained during the review of “Epazote (*Dysphania ambrosioides*): A phytochemical treasure with multiple applications: A review”.

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## Review

# Natural Products as Hepatoprotective Agents—A Comprehensive Review of Clinical Trials

Piotr Służały <sup>1</sup>, Paweł Paśko <sup>2</sup> and Agnieszka Galanty <sup>1,\*</sup>

<sup>1</sup> Department of Pharmacognosy, Jagiellonian University Medical College, Medyczna 9, 30-688 Cracow, Poland; piotr\_s-95@o2.pl

<sup>2</sup> Department of Food Chemistry and Nutrition, Jagiellonian University Medical College, Medyczna 9, 30-688 Cracow, Poland; p.pasko@uj.edu.pl

\* Correspondence: agnieszka.galanty@uj.edu.pl

**Abstract:** The hepatoprotective effects of natural products have been a significant focus in recent decades due to the growing demand for the help in the treatment of hepatic impairments. This review specifically delves into the findings of clinical trials involving 13 selected natural products, namely plants and their derived compounds (e.g., artichoke, berberine, and turmeric), algae (e.g., spirulina), probiotics, and other products like phospholipids and vitamin D. A literature search was performed in the Scopus database, PubMed, and Google Scholar, covering all articles found up to June 2024. Artichoke, berberine, chlorella, chicory, green tea, probiotics, phospholipids, schisandra, silymarin, spirulina, and vitamin D caused a decrease in liver enzymes, while for cinnamon and turmeric such an effect was either not observed or not convincing. The presented results indicate that some natural products might satisfactorily improve hepatic outcomes in NAFLD, NASH, and other liver disorders; however, further studies and metaanalyses are needed to clearly demonstrate their effectiveness.

**Keywords:** liver; herbal medication; hepatoprotective; clinical trials

## 1. Introduction

The liver is one of the most important organs of the human body, with a crucial role in physiological processes such as bile secretion, synthesis of vital proteins like albumins and fibrinogen, and also storage and metabolism. The latter role is especially critical in terms of drug metabolism within the body, impacting the safety, pharmacological effectiveness, and potential hepatotoxicity of medications. Despite all of those integral functions, hepatic impairments continue to become a major health concern worldwide [1]. Damage to hepatocytes, connective tissue, or the liver as an organ itself may be induced by autoimmune diseases (primary biliary, cirrhosis, or immune hepatitis) and biological factors (viruses, bacteria, and other organisms). The overuse of chemicals (e.g., thioacetamide, carbon tetrachloride, and ethanol) or drugs (e.g., acetaminophen, antituberculars, antiepileptics, and cytostatics) could also affect hepatic functioning [2]. Chronic liver diseases have become a main cause of mortality and morbidity over the last decade, with as many as two million annual deaths globally [3]. The common reasons for chronic liver disease are non-alcoholic fatty liver disease (NAFLD), hepatitis B and C, cirrhosis, and hepatocellular carcinoma which, as complications, contribute to liver-related mortality [4]. Metabolic risk factors for NAFLD include features of the metabolic syndrome that indicate increased cardiovascular risk. According to Bedogni et al. [5], 91% of those affected by NAFLD are overweight or obese [5]. Additionally, 55% of individuals with NAFLD also suffer from impaired glucose regulation or type 2 diabetes mellitus (T2DM), as noted by Ballestri et al. [6]. Other contributing factors may include hypertension, hyperlipidemia, family history, and the use of certain drugs such as tamoxifen, methotrexate, and diltiazem.

NAFLD is a public health problem with a high prevalence, directly impacting around 11–16% of the world population. This pathological syndrome is characterized by excessive

build-up of lipids in hepatocytes. The mechanism of this state is an imbalance between the input and output of free fatty acid metabolism in liver tissue [7]. Pathogenesis of NAFLD was recently linked to metabolic disturbances and changes in the glucose–insulin relation. Therefore, the name of NAFLD was re-evaluated to the newer name of metabolic (impairment)-associated fatty liver disease (MAFLD).

Despite great discoveries in modern pharmacology, no effective medication may cure completely or show an enormous improvement in hepatic functioning and the regeneration of hepatocytes [8]. The pharmacological treatment options for NAFLD include insulin sensitizers (metformin, rosiglitazone, and semaglutide), lipid-lowering drugs (clofibrate, gemfibrozil, and atorvastatin), angiotensin receptor blockers (telmisartan and candesartan), pentoxifylline, ursodeoxycholic acid, and antioxidants, but also herbal medicines [9]. A well-established therapy includes weight loss and dietary intervention that may significantly increase the effectiveness of medication treatments [10]. Recently, the treatment methods recommended by gastroenterology guidelines include lifestyle intervention as a holistic approach, drug treatment, and surgical procedures [11]. However, some natural products, both as drugs and as diet elements and dietary supplements, have been widely used as supports in liver functioning or in the prevention of liver diseases' development. The use of these natural products is based on their traditional uses, but the effectiveness of some of them have been examined in animal and human studies. However, the question arises of which natural product is worth choosing, in terms of its effectiveness in liver disorders. Thus, the aim of this review is to collect the data on the so far performed clinical trials of natural products, including plants, their derived compounds, and algae, microorganisms, or vitamins, and to critically compare their use in NAFLD and some other liver diseases.

#### Search strategy

A literature search was conducted in the Scopus database, PubMed, and Google Scholar, covering all articles found up to July 2024. Initially, the search term “liver and clinical trials and plants” was used, and then the term “liver and clinical trials and silymarin” was used in combination with the following: chicory, turmeric, artichoke, schisandra, berberine, vitamin D, green tea, spirulina, and probiotics. For example, “liver and clinical trials and silymarin” found 1708 articles. An additional criterion was introduced to include only English language results. After checking the titles and abstracts, 456 items were excluded due to being trials other than clinical and/or human trials or reviews. Only 289 were included with relevance to this review. Of the remaining 202 articles, a total of 84 were used for this review (Figure S1).

For the purposes of this review, the details of the studies performed are gathered in the appropriate tables in the Supplementary Material. To facilitate the understanding of the presented effects of the examined substances on liver functioning, different outcome measures are briefly characterized below.

#### Liver functioning outcome measurements

Determination of the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), or gamma-glutamyl transferase (GGT) can assist in identifying specific areas of the liver that may be experiencing damage and, based on their pattern of elevation, can aid in proper diagnosis. Elevations in ALT and AST in comparison to the elevations in ALP and bilirubin indicate hepatocellular disease [3]. Conversely, an elevation in ALP and bilirubin in comparison to ALT and AST would indicate a cholestatic pattern [2]. The activity of these enzymes is presented in the manuscript as a percentage difference between baseline parameters and post-intervention results in both the intervention and placebo groups.

The fatty liver index (FLI) is a comprehensive algorithm that incorporates body mass index (BMI), waist circumference (WC), GGT, and triglyceride (TG) levels. It has been shown to be an effective tool in predicting the presence of NAFLD, due to its strong correlation with both imaging and histological indicators of the disease [4].

The lipid accumulation product (LAP) is an index that combines WC and TG levels to assess lipid accumulation. Higher LAP results indicate a more severe liver condition [12].

The hepatic vein waveform typically shows a triphasic pattern, characterized by retrograde flow during atrial contraction and two antegrade pulses during both ventricular systole and ventricular diastole [7].

A liver ultrasound is a non-invasive and straightforward diagnostic tool used to assess for a variety of liver conditions, such as cirrhosis, fatty liver disease, cancer, and other abnormalities: adipose accumulations within the hepatic organ, indicative of potential steatosis, fibrous tissue development or sclerosis within the hepatic organ, potentially signaling cirrhotic changes, abnormal growths within the liver, possibly suggestive of malignancies or infectious processes, or manifestations of hepatitis, inclusive of hepatic inflammation [9]. These include (i) the hepatorenal index, a semiquantitative ultrasonographic examination to estimate liver steatosis, calculated by dividing the mean brightness of the liver by the mean brightness of the kidneys; (ii) doppler flow, comprising the hemodynamic parameters of the portal vein, including flow pressure, flow volume rate, and wall shear stress (WSS); (iii) the liver–spleen (L/S) attenuation ratio, of significant value in the diagnostic assessment of hepatosteatosis due to its inherent benefits, including its ability to be measured within the same cross-sectional imaging study and its relative insensitivity to the presence of most systemic diseases; (iv) the diameter of the portal vein, which typically falls within a range of approximately 7–15 mm; and (v) the liver fatty content, where ultrasound technology is utilized to measure the attenuation of ultrasound waves in the liver for the purpose of assessing hepatic fat content [11].

## 2. Plants and Compounds Derived from Plants

### 2.1. Artichoke

Artichoke (*Cynara cardunculus* L.) is a plant widely consumed in the Mediterranean diet. Pharmacological effects are related to the presence of antioxidants, such as cynarin, chlorogenic acid, caffeic acid and its derivatives, and the volatile sesquiterpene and flavonoids, including the glycosides of luteolin [13]. The hepatoprotective mechanism is connected to cholesterol reduction, as artichoke is well known for decreasing the reactive oxygen species level, lipid peroxidation, protein oxidation, the oxidation of LDL-cholesterol, and causing an increase in glutathione peroxidase activity [14,15].

A total of eight randomized clinical trials were conducted to evaluate the impact of artichoke on liver enzymes in patients with NAFLD ( $n = 299$ ) or hypercholesterolemia ( $n = 267$ ). The research took place in various countries including Iran, Italy, France, and Germany. In the case of patients with NAFLD, the artichoke extract was administered in a range, from 300 to 2700 mg/day, over a period of 2–4 months, and in one case the artichoke was combined with a bergamot polyphenolic fraction (Table S1). The results indicated a significant decrease in liver parameters (ALT and AST), with a clear correlation between the dosage used and the observed effectiveness, demonstrating that the higher doses of the artichoke extract were more effective in inducing hepatic enzyme changes compared to the lower ones. Moreover, liver ultrasonography showed a significant improvement in terms of fat accumulation in the liver in two artichoke groups [16]. Doppler sonography ultimately revealed that analogous advantageous effects were unveiled due to the increase in hepatic vein flow, the enlargement of the liver size, and the reduction in portal vein diameter [17]. The beneficial effects of the artichoke combined with the bergamot polyphenolic fraction were manifested as an improvement in the hepatorenal index and liver brightness [18].

For patients with hypercholesterolemia, artichoke extract alone, but also other plant extracts and vitamins included in the combination, was administered in a range, from 20 to 450 mg/day, for a period of 4 to 8 weeks. However, the results of these studies are inconclusive, as two trials revealed significant changes observed in liver enzymes [14,19], while the other two showed the opposite effects [15,20]. Interestingly, in one of the studies, despite no observed effect on liver enzymes, the NAFLD indexes (lipid accumulation

product and fatty liver index) were significantly improved [20]. The highest daily dose used in the studies was 2700 mg of the artichoke extract [21].

## 2.2. Berberine

Berberine is a quaternary isoquinoline alkaloid found in an ancient Chinese herb, *Coptis chinensis* Franch., and *Berberis aristata* DC., which has been used to treat diabetes for many years [22]. The efficacy of berberine in liver-related issues is connected to its cholesterol-lowering effect by the induction of the expression of LDL-cholesterol in hepatocytes' post-transcription mRNA stabilization to encoding receptors [23]. Berberine has been proven to significantly reduce hepatic fat content in rats with NAFLD by decreasing methylation of the MTP (microsomal triglyceride transfer protein) promoter and mitigating the hepatic steatosis caused by a high-fat dietary regimen in rodent models [24].

In total, five randomized clinical trials (Table S2) conducted in China, the USA, and Iran evaluated the impact of berberine on liver enzymes in patients with NAFLD and NASH [22–26]. A total of 478 patients aged 40–54 with liver impairment participated in the studies, which included assessments of ALT, AST, and GGT levels. Berberine dosages ranged from 1 to 6.25 g/day, with intervention periods lasting from 6 to 18 weeks. The results demonstrated a significant improvement in liver parameters, with a clear dose-response relationship observed in one study comparing 1 and 2 g doses. Higher doses of berberine were associated with greater reductions in hepatic enzyme levels. Additionally, a study by Yan et al. [23] showed a significant reduction in liver fat, which was assessed by a proton magnetic resonance spectroscopy, in the berberine group compared to the placebo and reference groups [23]. A greater reduction in liver fat content, measured by spectroscopy, was also confirmed by Harrison et al., with a dose-dependent effect noted for berberine compared to the placebo group [25].

## 2.3. Chicory

Chicory (*Cichorium intybus* L.) has been traditionally used for the treatment of liver diseases for thousands of years. It has antioxidant properties which are likely to be due to the presence of polyphenolic compounds [27,28]. Many trials have suggested that inulin, which is 40% present in chicory root extract, is effective in enhancing the expression of iron genes, enzymes, and ferritin in enterocytes, which corresponds to a reduction in serum ferritin and a change in liver enzyme levels [29].

A total of 374 individuals with NAFLD or cirrhosis or hepatobiliary issues were studied (Table S3) to evaluate their liver parameters following the consumption of chicory [27–35]. One study from India and eight studies from Iran were included in the analysis. Six studies focused solely on chicory in various forms, such as a water-plant, chicory leaf, a powder extract of the root, and powdered seeds. The interventions involved different doses of the mentioned extracts over a period ranging from 4 weeks to 6 months. Only one of the nine studies did not show significant improvements in liver parameters, with 61 NAFLD patients, where the intervention group received the lowest dose of chicory at 500 mg/day of powdered chicory root, while both the intervention and placebo groups were advised to make lifestyle modifications to meet dietary requirements. The results did not demonstrate significant changes between the intervention and placebo groups [30]. Overall, improvements in liver parameters may be attributed to lifestyle changes and dietary adjustments.

Outstanding results were noted in a study by Ghaffari et al., where the intervention included turmeric and chicory, alone or in combination. The highest impact in ALT serum levels was seen in the turmeric group at −21.1%, then in the group taking a combination of both substances at −17.5%, and the lowest was seen in the chicory group at −14.0%, vs. the placebo group at −3.5% [31]. A significant reduction in liver parameters was noted by Huseini et al., in which chicory was one of eight herbal ingredients (Liv-52). Unfortunately, it cannot be clearly stated if the effect was related to chicory or the synergistic impact of the other ingredients [32]. Another study was conducted by Parveen et al. on 30 subjects

with hepatobiliary disease where the intervention included the combination of chicory and *Solanum nigrum*, but with no placebo group. The results showed significant improvements in liver parameters [28]. Significant decreases in the grade of sonographic examinations were assessed in a study by Sheybani et al. compared to the placebo group [33]. Marzaban et al. found a similar outcome in terms of the grade of NAFLD [27].

#### 2.4. Cinnamon

Cinnamon is widely used as a spice in food preparation and herbal remedies for some diseases and inflammation. This herbal product is found in candy, toothpaste, and perfumes. Cinnamaldehyde is the main compound of cinnamon and can regulate immunological function via anti- and pro-inflammatory gene expression. This has been confirmed in many in-vitro and in-vivo studies. Traditionally, it is used as antioxidant and for the regulation of lipid profiles and glucose [36].

A sole, double-blind RCT study describing the impact of cinnamon (*Cinnamomum verum* J. Presl) on the liver was performed by Askari et al. [36] on 45 patients diagnosed with NAFLD in Iran. The researchers aimed to investigate a potential association between the daily consumption of 1.5 g of concentrated cinnamon extract ( $n = 23$ , 10 F, 13 M mean age 44.8, and BMI 29.9) over a 12-week period compared to a placebo group ( $n = 22$ , 13 F, 9 M mean age 45.4, and BMI 30.3). The trial focused on assessing the levels of liver enzymes such as ALT, AST, and GGT. The results showed a notable improvement in all liver parameters in the group that received the intervention. The ALT levels decreased by 37.25% compared to 2.07% in the placebo group, AST levels decreased by 36.73% compared to 2.77% in the placebo group, and GGT levels decreased by 32.47% compared to 3.46% in the placebo group [36].

#### 2.5. Curcumin

Curcumin is a phenolic compound found in the roots of turmeric (*Curcuma longa* L.), a plant widely grown in India, China, and Indonesia [37]. Curcumin has been intensively studied for its anti-inflammatory, lipid-regulation, anti-arthritis, antioxidant, immunomodulatory, antidepressant, and anti-cancer potential. However, the health benefits of the compound in humans are problematic due to its low bioavailability [38].

A total of 575 patients with NAFLD and/or liver cirrhosis were included in the analysis. The studies (Table S4) were conducted in Italy and Iran [37–44]. Most of the studies were conducted using curcumin. Only one study included turmeric at a daily dosage of 2 g, with the remaining studies focusing on the standardization of curcumin. The most recent formulations of curcumin that have been studied include synacurcumin (80 mg), an amorphous dispersion curcumin formulation (equivalent to 70 mg of curcumin), 800 mg of phytosomal curcumin, and a combination of 500 mg of curcuminoids with 5 mg of piperine, which is used to enhance the bioavailability of curcuminoids. The majority of the studies (seven out of eight) reported improvements in liver parameters. The follow-up period of the studies ranged from 4 weeks to 6 months.

Hepatic fibrosis was assessed as a parameter by Saadati et al., with a significant reduction in the curcumin group only [38]. Ultrasonographic examination in another study demonstrated an improvement of the reduction in liver fat content in 78.9% of participants in the curcumin group, compared to only 27.5% in the placebo group. No instances of increased liver fat content were observed in the curcumin group, whereas 17.5% of individuals in the placebo group experienced an increase in liver fat content [39]. The findings by Cicero et al. demonstrated similar outcomes in relation to the parameters of fatty liver index and liver steatosis [40]. Jazayeri-Tehrani et al. indicated that the administration of nano-curcumin to overweight/obese patients with NAFLD resulted in improved liver function and a reduction in fatty liver severity [41]. Nouri-Vaskeh et al. discovered that curcumin supplementation had positive impacts on disease activity scores and the severity of cirrhosis [37]. Panahi et al. [42] elucidated that the administration of curcuminoids together with piperine resulted in a considerable reduction in the severity



of NAFLD, a phenomenon that was found to be statistically distinct in comparison to the placebo group. Moreover, it was observed that individuals in the placebo group did not exhibit any alterations from their initial baseline measurements [42]. The ultrasonographic findings of Panahi et al. [43] showed a significant improvement of NAFLD severity in 75.0% of subjects in the curcumin group, compared to only 4.7% in the control group. Additionally, the occurrence of increased liver fat content was lower in the curcumin group at 4.5%, while 25.6% of subjects in the placebo group experienced an increase. Analysis between the groups demonstrated notable enhancements in hepatic vein flow, portal vein diameter, and liver volume in the curcumin group compared to the placebo group [44].

## 2.6. Green Tea

Green tea is defined as the unfermented leaves of *Camellia sinensis* (L.) Kuntze. It is the second most popular drink, after water, especially in Southeast Asia. Consumption of tea is related to flavanols and other polyphenols known as catechins, but also caffeine, which may be health beneficial [45]. Epigallocatechin-3-gallate (EGCG) is the major present catechin in green tea [46]. The medical properties are as follows: cytotoxic, anti-diarrheal, anti-microbial, antioxidative and anti-inflammatory, anti-obesity, and anti-diabetic [47].

A total of 253 patients diagnosed with NAFLD and NASH were administered green tea catechin at dosages ranging from 550 to 1080 mg per day, or a green tea extract of 500 mg standardized for its epigallocatechin gallate (EGCG) content (31.43% to 52.6%), for a duration of 12 to 24 weeks (Table S5). These patients were from two different locations in Japan, as well as from Iran and Pakistan [48]. The impact of the treatment was assessed based on various liver parameters, including ALT levels, as measured in all five studies, as well as AST in four studies, GGT in one study, and ALP in one study. Results across all clinical trials consistently showed a significant decrease in these liver parameters. It is important to note that a study conducted by Pezeshki et al. (2016) revealed a reduction in hepatic outcomes among placebo groups, suggesting a potential correlation with lifestyle modifications [45].

Fukuzawa et al. shown that green tea catechins, in combination with structured dietary plans and exercise regimens, can improve key anthropometric parameters in ultrasonographic findings, such as the ratio of visceral fat to subcutaneous fat (V/S) area, as well as the liver-to-spleen (L/S) ratio. This suggests that these interventions may help to prevent the progression of NASH by utilizing their antioxidant and anti-inflammatory properties to reduce oxidative stress in patients with NASH [46]. Hussain et al. [48] revealed a significant 67.5% reduction in fatty liver changes in ultrasounds among participants who consumed green tea extract, in comparison to those who received a placebo showing only a 25% regression. This suggests that green tea extract may play a promising role in inhibiting the advancement of NAFLD [48]. Sakata et al. [49] discovered that individuals who consumed a high-density catechin supplement experienced a significant decrease in body fat compared to those taking a placebo or low-density catechin supplement over a period of 12 weeks. Furthermore, all participants in the high-density catechin group exhibited a notable improvement in their liver-to-spleen CT attenuation ratio as compared to those in the placebo and low-density catechin groups after the same duration of consumption [49].

## 2.7. Schisandra

Schisandra (*Schisandra chinensis* (Turcz.) Baill.) fruits are traditional East Asian medicine used in obesity and diabetes [50]. The fruits are rich in lignans called schisandrins with antioxidative, anti-inflammatory, and cytotoxic properties [51]. Schisandrin B reduced hepatic lipids in rat studies [52]. The probable mechanism of action of schisandra fruits involves the inhibition of adipogenesis in preadipocyte cells, as noted in the in vivo studies [50].

A total of 100 patients (Table S6) with mild liver injury or dysfunction were included in two studies to assess the impact of schisandra on hepatic outcomes. The trials were

conducted in Indonesia and Taiwan. Patients were administered a schisandra extract (7.5 mg daily) or schisandrin B (0.24–0.48 mg) combined with sesamin.

The first study, conducted by Ardiyanto et al., found that the schisandra extract was just as effective as a traditional herbal medicine (used in the placebo group) in treating liver disturbances, as evidenced by confirmed results in ALT and AST levels [51].

The second study showed a significant decrease in liver parameters among patients in the intervention group who were given a combination of schisandrin B and sesamin. Moreover, a marked improvement in the ultrasonographic examinations of fatty liver and liver function was observed. However, it should be noted that this study did not establish the effectiveness of schisandrin B on its own, but rather in combination with sesamin [52].

## 2.8. Silymarin

Silymarin is a flavonolignan fraction of milk thistle (*Silybum marianum* (L.) Gaertner), comprising mainly silybin, silydianin, silychristin, and isosilybin. The seeds of milk thistle have a long history of use in liver disorders [53]. The use of milk thistle (silymarin) is believed to have several potential benefits in the field of hepatology. These include preventing the entry of toxins such as alcohol, carbon tetrachloride, and heavy metals into liver cells, stimulating protein synthesis for liver regeneration, acting as an antioxidant to scavenge free radicals, and modulating the immune response. Research studies have shown hepatoprotective effects of silymarin in cases of fatal fulminant hepatic failure caused by *Amanita phalloides* mushroom poisoning in both animal and human subjects, even when administered after exposure. While some randomized controlled trials have reported inconclusive results, particularly in cases of alcoholic liver disease, chronic hepatitis B (HBV), or hepatitis C (HCV) infections, further research is needed to fully understand the potential benefits of milk thistle/silymarin supplementation in these conditions [54].

A total of 676 patients diagnosed with various liver conditions, including NAFLD, NASH, acute/chronic hepatitis, cirrhosis, and other liver disorders, were included in the studies (Table S7) to evaluate the impact of silymarin on liver health outcomes [53–60]. The patients ranged in age from 29 to 63 years old. The studies were conducted in multiple countries, including Iran, Australia, Spain, Egypt, Finland, and Denmark. Patients were administered varying dosages of milk thistle extract—with some standardized for silymarin amounts ranging from 140 to 600 mg daily, with a standard dose of 420 mg for a follow-up period of 4 weeks to 12 months. While three out of the eight studies did not demonstrate significant improvements in liver outcomes, one study conducted by Fried et al. (2012) revealed that a higher dose of silymarin, specifically 720 mg daily, was more effective in reducing liver parameters compared to the standard 420 mg dosage [55]. The histological examination conducted by Salmi and Sarna (1982) revealed a statistically significant improvement in the silymarin group compared to the placebo group in terms of fatty liver transformation and the score of hepatitis [56].

## 3. Algae and Probiotics

### 3.1. Chlorella

Chlorella (*Chlorella vulgaris* Beijer.) is a unicellular green algae known worldwide as a functional food and used as a traditional medicine in Asia [61]. It is rich in choline,  $\beta$ -carotene, and amino acids. Numerous essential vitamins and minerals are present in chlorella, including thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folic acid, cobalamin, ascorbic acid, tocopherols, sodium, potassium, calcium, magnesium, phosphorous, copper, zinc, manganese, iodine, and iron. Its bioactive compounds, mainly pigments, include chlorophyll, beta-carotene, astaxanthin, canthaxanthin, violaxanthin, lutein, and pheophytin, essential for many bioactivities [17]. In animals, many health-beneficial effects were found, such as anti-microbial, cytotoxic, and anti-diabetic effects, but also improvements in liver function were noted. However, there has been a limited number of trials on human subjects [62]. The mechanism underlying the improvement of hepatic outcomes might be related to the weight loss effects of chlorella, observed as a

decrease in hepatic triglyceride content and gluconeogenesis, which eventually results in a reduction of liver parameters [17].

In a total of 271 patients diagnosed with NAFLD or hepatitis C, the interventions involved the administration of chlorella in powdered tablets, ranging from 1 to 3 g daily for a period of 8 to 12 weeks. The majority of the studies (Table S8), four out of five, were conducted in Iran, with one study originating from the USA [63]. All five studies demonstrated overall enhancements in liver parameters, with the exception of Panahi et al. (2012) where a placebo group administered with metformin showed a greater decrease in ALT and ALP serum levels compared to the chlorella intervention group [17]. It is important to note that only AST levels showed improvement in this particular study. Additionally, Ebrahimi et al. (2014) conducted a notable study which exhibited some reduction in liver parameters in the placebo group (which received only vitamin E supplementation) [61]. A study by Azocar and Diaz (2013) showed a statistical decrease in HCV viral load. The HCV RNA levels were confirmed in 69.23% of the participants [63]. Other studies have not assessed physical liver function [17,61,62,64,65].

### 3.2. *Spirulina*

*Spirulina* (*Arthrospira platensis*) is a green–blue, multicellular microscopic algae mostly found in alkaline lakes. This filamentous cyanobacterium is cultivated in human-controlled conditions for global consumption [66]. It has been used for thousands of years but recently is becoming more popular, as it is a rich source of essential nutrients including vitamins such as thiamine, riboflavin, niacin, cobalamin, ascorbic acid, cholecalciferol, and toco-pherol; minerals including potassium, calcium, iron, magnesium, phosphorus, selenium, sodium, and zinc; pigments (e.g., chlorophyll A, xanthophylls,  $\beta$ -carotene, zeaxanthin, canthaxanthin, diatoxanthin,  $\beta$ -cryptoxanthin, C-phyco- cyanin, and allophyco- cyanin); and enzymes (such as lipase), as well as eight crucial amino acids. The ratio of saturated palmitic acid in the total fatty acids is 25.8%, while the percentage of  $\gamma$ -linolenic acid in the total fatty acids is 40.1%. *Spirulina* serves as a valuable dietary supplement to address essential fatty acid deficiencies and provide antioxidants (including chlorophyll, phyco- cyanin pigments, and phenolic compounds which equate to 26.64 mg of gallic acid per gram of extract) [67]. *Spirulina* has revealed positive outcomes in trials of NAFLD, diabetes, arterial hypertension, and hypercholesterolemia [65].

A total of 128 patients with NAFLD or chronic viral hepatitis were included in a study (Table S9) analyzing the effect of spirulina on liver function. Two studies were conducted in Iran, with additional studies from Romania, Mexico, and Greece. *Spirulina* doses ranged from 1 to 4.5 g in the form of powdered tablets, taken daily for 4 weeks to 6 months. Only two out of the five studies included a placebo group. Overall, significant improvements in hepatic liver enzymes were reported in all studies, with the exception of the study by Baicus and Tănăsescu (2002) which focused on patients with viral chronic hepatitis (with etiology of infection with hepatitis virus B or C), potentially indicating different results compared to those with NAFLD [68]. Notably, a case report from Mexico involving three patients with NAFLD demonstrated significant impacts on liver parameters, observed as a notable enhancement in liver ultrasonography. All patients exhibited an improvement in liver ultrasonography related to NAFLD. Two patients showed a decrease in parenchyma heterogeneity and the third patient experienced a complete resolution of the “bright liver” phenomenon. However, the results can only be treated as preliminary. The authors opted not to perform liver biopsies due to the high risk involved for ambulatory patients and the limited technical and medical resources available in the laboratory to manage potential complications [69]. On the contrary, the study by Mazokopakis et al. (2014) did not reveal any significant alterations in sonographic results within the two groups under observation [67].

### 3.3. Probiotics

Probiotics are traditionally described as a variety of bacteria in the intestine with a full spectrum of health benefits for the host. The mechanism of how those microbes work in patients with NAFLD is not fully understood [70]. It is suggested that it might be connected to the interaction of eradicating pathogenic bacteria in the intestine [71]. Potential mechanisms of action related to liver disease involve the reduction of circulating levels of pro-inflammatory mediators, such as TNF- $\alpha$ , IL-1 $\beta$ , or IL-6. Additionally, there may be a decrease in hepatocyte ballooning and the development of liver lobular fibrosis [72]. Intestinal dysbiosis is called intestinal bacterial overgrowth (SIBO). This acts via the blocking of adhesion sites, degradation of toxin receptors, and absorption of gut-derived lipopolysaccharides [73].

A total of 682 patients diagnosed with NAFLD, NASH, minimal hepatic encephalopathy, or obesity-related liver disease in children were included in the reviewed studies, evaluating the efficacy of probiotics and prebiotics on liver parameters. The studies (Table S10) originated from multiple countries, with three from Iran, two from India and Italy, and single ones from Spain, Egypt, Ukraine, the USA, Brazil, and Hong Kong [64,70–81]. The interventions in the studies varied, with three utilizing a single strain of bacteria, including *Lactobacillus rhamnosus* (12 billion CFU) (dsm2 21690), *L. acidophilus* (2 billion CFU) (DSM B3208, DSM 24735), or *Bifidobacterium longum* (2 billion CFU) (DSM 24736), as well as combinations such as *L. bulgaricus* and *Streptococcus thermophilus* (500 million CFU) (DSM 24731) or various multi-strain formulations ranging from 15 million to 112.5 billion CFU.

The notable research conducted by Javedi et al. (2017) indicated a more pronounced decrease in liver parameters within the probiotic plus prebiotic group when compared to the probiotic group alone [72]. In addition, a study by Malaguarnera et al. (2012) revealed a considerable decrease in liver parameters within the placebo group, which the authors attributed to lifestyle interventions within both groups [75].

Subsequent liver biopsies were conducted by Duseja et al. (2019) one year post-initiation in a cohort of 10 patients (52.6%) within the probiotic group and five patients (25%) within the placebo group. Significant enhancements in hepatocyte ballooning, lobular inflammation, and NAS score were observed at the one-year mark in the probiotic group compared to baseline levels. Furthermore, significant improvements were noted in the NAS score, hepatocyte ballooning, and hepatic fibrosis in the probiotic group when compared to the placebo group [76]. Famouri et al. (2017) discovered that after their trial, normal liver sonography was reported in 17 (53.1%) of the patients in the intervention group and five (16.5%) of the patients in the placebo group. This demonstrates the beneficial effect of probiotic supplementation [77]. The research conducted by Javadi et al. (2017) demonstrated that the use of a probiotic (capsules containing *B. longum* and *L. acidophilus* at a dosage of  $2 \times 10^7$  CFU per day), a prebiotic (group 2, high-performance inulin administered at a dosage of 10 g per day), and a combination of the probiotic and prebiotic (group 3) led to improvements in the severity of fatty liver in patients with NAFLD [72]. Malaguarnera et al. (2012) demonstrated that the combination of *B. longum* supplementation with prebiotics and lifestyle modifications significantly reduced steatosis and the NASH activity index compared to lifestyle modification alone [75]. After the intervention, Wong et al. (2013) found that liver fat decreased by a mean of 7.7%, as observed through proton-magnetic resonance spectroscopy. In contrast, the usual-care group showed a static hepatic fatty content with a mean reduction of only 1.0% [78]. On the contrary, Monem (2017) demonstrated that probiotic treatment did not lead to any notable changes in abdominal ultrasound results [79].

## 4. Other Natural Products

### 4.1. Essential Phospholipids

Phospholipids are the main components of mammalian cells and lipoproteins [82]. They are necessary for the formation, regeneration, and movement of membrane structures and the restoration of membranes' structure and fluidity, the inhibition of fibrotic processes,

and the modification of lipid metabolism, and therefore have anti-inflammatory and antioxidant properties [83]. Essential phospholipids (EPLs) are the purified fraction obtained from soybeans and are widely used in liver diseases [84].

In total, 705 patients with NAFLD and NASH were enrolled in trials involving essential phospholipids for the improvement of liver enzymes (Table S11) [82–89]. Among these trials, only three out of eight were randomized controlled trials with a placebo group, while the rest were open-label studies. The participants in these studies ranged in age from 41 to 59, with a BMI between 28.2 and 29.3. The studies were conducted in various countries including China (three studies), Poland, India, Russia, the United Arab Emirates, and Sweden.

All studies utilized the same dosage of 1800 mg of essential phospholipids per day, administered in divided doses. The duration of follow-up ranged from 4 to 48 weeks. Results from all studies consistently showed a decrease in liver parameters. One study by Yin and Kong (2000) included a comparative analysis with metformin [85]. However, the effectiveness of different dosages could not be assessed as all studies used the same dosing regimen.

The ultrasound studies conducted by Poongothai et al. (2005) demonstrated that hepatic echotexture improved in 54.5% of the study subjects following EPL treatment. Conversely, no change was observed in 40.9% of the participants, and only one patient (4.5%) experienced a worsening of hepatic echotexture [86]. In a study conducted by Gonciarz et al. (1988), it was demonstrated that EPL exhibited significant effectiveness in reducing liver size, likely due to its ability to decrease liver fat content. This finding was corroborated by histological evaluations of post-treatment biopsies, which indicated the absence of fatty infiltration in four patients from the Essentiale Forte group exclusively [82].

#### 4.2. Vitamin D

Cholecalciferol (vitamin D) is crucial for maintaining phosphate and calcium balance [90]. Those subjects affected by D-deficiency are more likely to develop impaired glucose tolerance and increase the amount of free fat in their liver which is directly linked to the development of NAFLD [90,91]. In recent decades, research has conclusively demonstrated the role of vitamin D in modulating various immune–inflammatory and metabolic processes [92]. Subsequently, there has been a focus on studying the interaction between the active form of vitamin D—1,25-dihydroxy-vitamin D—and the vitamin D receptor (VDR) in relation to disruptions in metabolic pathways within various organs and tissues. This includes but is not limited to the skeletal muscle, adipose tissue, and liver, which are crucial players in metabolic regulation [93].

A total of 461 individuals with NAFLD were included in the studies to assess the effectiveness of vitamin D on liver parameters (Table S12) [90–94]. The dosage of vitamin intake ranged from 800 IU daily to 50,000 IU weekly when taken orally, or from 200,000 IU to 600,000 IU in a single intramuscular injection when administered parenterally. The average age of the participants ranged from 38.3 to 54, with a BMI range from 27.0 to 31.2. The studies were conducted in Iran, Egypt, and India, with follow-ups ranging from 10 weeks to 12 months. Four out of five studies demonstrated statistical improvements in liver function, with no observed correlation between dosage and effectiveness.

Notably, a study by Shidfar et al. (2019) highlighted the synergistic effect of vitamin D combined with calcium on liver enzymes ALT and AST compared to vitamin D alone. This suggests that a combination of both substances may be beneficial in maintaining optimal levels of vitamin D and calcium in the body [92].

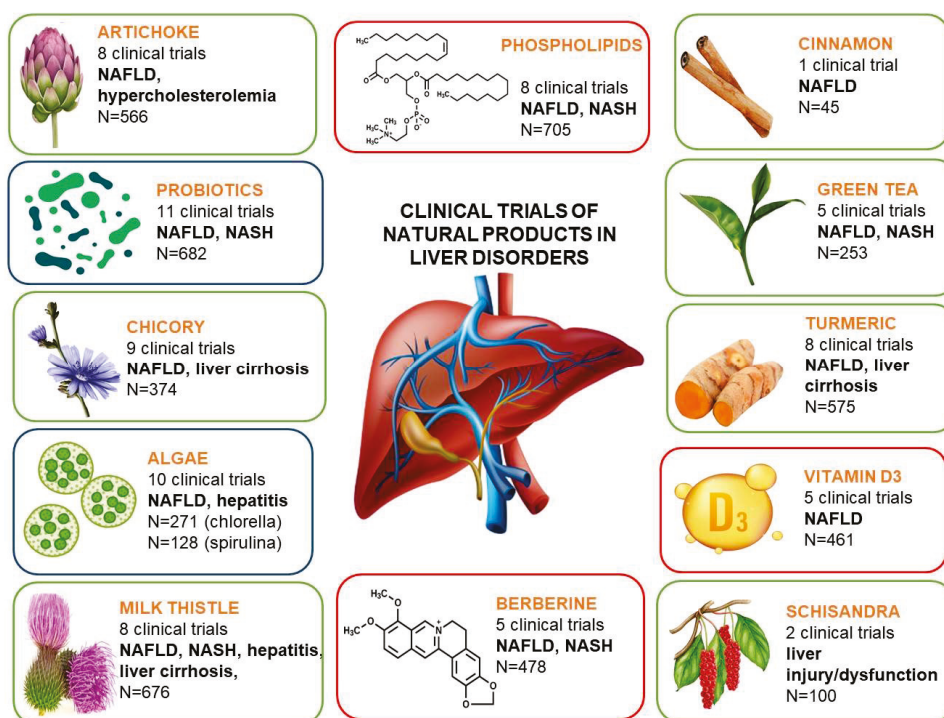
Sakpal et al. (2017) discovered that there was no variance in the baseline level of steatosis and hepatic fibrosis stage between the two groups who received either vitamin D or a placebo [93]. Gad et al. found that a single monthly intramuscular dose of 200,000 IU cholecalciferol effectively improved laboratory and fibroscan parameters of the liver in patients with NAFLD [91].



## 5. Discussion

Our review comprised thirteen natural products often used in supporting liver disorders as herbal drugs or dietary supplements. The presented results indicate that some of the presented natural products might satisfactorily improve hepatic outcomes in NAFLD, NASH, and other liver disorders. None of the studies indicated a significant toxic effect of these non-synthetic remedies on liver function.

Apart from the well-known natural products, like silymarin, artichoke, or essential phospholipids, often used by patients with liver disorders, some new, often surprising candidates for liver support can be indicated, based on the results of the studies cited in this review (Figure 1). These may particularly include the representatives of algae, namely chlorella and spirulina, but also the probiotics. The latter candidate seems to be of the highest interest, as the clinical studies on probiotic use in liver disorders comprised almost 600 patients/participants (as compared to the often-used silymarin, with 676 participants), and were properly designed, which could be a solid evidence-based argument for their use. The studies concerning algae, despite the smaller number of participants, revealed interesting potential of both chlorella and spirulina in supporting liver disorders, and these studies deserve continuation to deliver more clear evidence.



**Figure 1.** Summary of the clinical trials of natural products presented in this review.

The studies presented in this review have some limitations. Firstly, using hepatic parameters such as ALT, AST, and ALP as direct markers for NAFLD diagnosis is not obvious, and there are no other effective measure outcomes to use in clinical trials. The number of studies performed and, in particular, the sizes of the groups included within the studies were relatively low or not satisfactory. Additionally, some studies lacked a control/placebo group, while in a few the reference group was receiving a drug affecting the metabolism (e.g., metformin), which might have disturbed the final results and conclusions drawn. Moreover, the majority of the studies were carried out in Iran; therefore, the effect of polymorphism was not investigated. The effect of sex was not investigated in this review because most trials have not quantified participants by male or female. Concerning the forms and doses used in the clinical trials cited, the direct comparison of the results for particular natural products was difficult, due to the high diversity of the examined products, e.g., an extract, standardized extract, pulverized natural product, or an isolated

fraction containing the dominant compounds. This was mainly observed for artichoke, chicory, and silymarin, where the unification of the products used in the studies needs improvement in the future. In contrast, the studies concerning essential phospholipids were easier to compare, as the dose used was the only difference between the studies. On the other hand, among the studies performed on essential phospholipids, the vast majority (six out of eight) were open label studies without a control group, which impairs their credibility.

## 6. Conclusions

In conclusion, the results of the studies included in this review indicate the improvement in some liver outcomes' measurements after the use of artichoke, berberine, chlorella, chicory, green tea, probiotics, phospholipids, schisandra, silymarin, spirulina, and vitamin D in liver injury and NAFLD. In contrast, other studies with cinnamon and turmeric have not significantly changed liver enzymes in response to herbal supplementation. However, it is important to underline that these studies refer to the effects of the mentioned products in patients with already identified liver diseases, while their hepatoprotective or perhaps hepatoprophylactic potential has not been assessed during the actual exposure to hepatotoxic agents. These results thus need confirmation in future larger-scale meta-analyses to seek the optimally effective doses of the examined natural products on liver enzymes, with longer supplementation periods, with NAFLD of varying severities, and with the assessment of other liver parameters (liver size, hepatic steatosis, and hepatic fibrosis).

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/plants13141985/s1>; Figure S1: Flowchart of the search strategy; Table S1: Clinical trials of hepatoprotective effect of artichoke; Table S2: Clinical trials of hepatoprotective effect of berberine; Table S3: Clinical trials of hepatoprotective effect of chicory; Table S4: Clinical trials of hepatoprotective effect of turmeric/curcumin; Table S5: Clinical trials of hepatoprotective effect of green tea; Table S6: Clinical trials of hepatoprotective effect of schisandra; Table S7: Clinical trials of hepatoprotective effect of milk thistle/silymarin; Table S8: Clinical trials of hepatoprotective effect of chlorella; Table S9: Clinical trials of hepatoprotective effect of spirulina; Table S10: Clinical trials of hepatoprotective effects of probiotics; Table S11: Clinical trials of hepatoprotective effect of phospholipids; Table S12: Clinical trials of hepatoprotective effect of vitamin D.

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## Review

# The Wild Carrot (*Daucus carota*): A Phytochemical and Pharmacological Review

Jana Ismail <sup>1</sup>, Wassim N. Shebaby <sup>1,\*</sup>, Joey Daher <sup>2</sup>, Joelle C. Boulos <sup>3</sup>, Robin Taleb <sup>4</sup>, Costantine F. Daher <sup>4,5</sup> and Mohamad Mroueh <sup>1</sup>

<sup>1</sup> Pharmaceutical Sciences Department, School of Pharmacy, Lebanese American University, Byblos 1102-2801, Lebanon; jana.ismail@lau.edu (J.I.); mmroueh@lau.edu.lb (M.M.)

<sup>2</sup> Gilbert and Rose-Marie Chagoury School of Medicine, Lebanese American University, Byblos 1102-2801, Lebanon; joey.daher@lau.edu

<sup>3</sup> Institute of Pharmacy and Biomedical Sciences, Department of Pharmaceutical Biology, Johannes Gutenberg University, 55128 Mainz, Germany; joboulos@uni-mainz.de

<sup>4</sup> Department of Natural Sciences, School of Arts and Sciences, Lebanese American University, Byblos 1102-2801, Lebanon; robin.taleb@lau.edu.lb (R.T.); cdaher@lau.edu.lb (C.F.D.)

<sup>5</sup> Alice Ramez Chagoury School of Nursing, Lebanese American University, Byblos 1102-2801, Lebanon

\* Correspondence: wassim.shebaby@lau.edu.lb

**Abstract:** *Daucus carota* L., a member of the Apiaceae family, comprises 13 subspecies, with one being cultivated (*D. carota* L. ssp. *sativus* (Hoffm.) Arcang.) and the remaining being wild. Traditionally, the wild carrot has been recognized for its antilithic, diuretic, carminative, antiseptic, and anti-inflammatory properties and has been employed in the treatment of urinary calculus, cystitis, gout, prostatitis, and cancer. While extensive literature is available on the phytochemical, pharmacological, and therapeutic evaluations of the cultivated carrot, limited information has been published on the wild carrot. A thorough search was conducted on the phytochemical composition, folk-medicine uses, and pharmacological properties of wild carrot subspecies (*Daucus carota* L. ssp. *carota*). Various electronic databases were consulted, and the literature spanning from 1927 to early 2023 was reviewed. Thirteen wild *Daucus carota* subspecies were analyzed, revealing over 310 compounds, including terpenoids, phenylpropanoids, flavonoids, and phenolic acids, with 40 constituting more than 3% of the composition. This review also highlights the antioxidant, anticancer, antipyretic, analgesic, antibacterial, antifungal, hypolipidemic, and hepato- and gastroprotective properties of wild carrot subspecies. Existing in vitro and in vivo studies support their traditional uses in treating infections, inflammation, and cancer. However, further research on other subspecies is required to confirm additional applications. Well-designed preclinical and clinical trials are still necessary to establish the safety and efficacy of wild *Daucus carota* for human use.

**Keywords:** *Daucus carota*; wild carrot; terpenes; anticancer; anti-inflammatory; antimicrobial

## 1. Introduction

*Daucus carota* belongs to the family Apiaceae or Umbelliferae and is part of the *Daucus* genus, which is a polymorphic taxon of multiple species and subspecies [1]. This family is characterized by aromatic, flowering, and celery/parsley-like plants that include edible as well as toxic species. *Daucus carota* L., commonly known as carrot, is classified into twelve subspecies; *D. carota* ssp. *sativus* (Hoffm.) Arcang. (the most well-known variety) and *D. carota* ssp. *boissieri* (Schweinf.) H. A. Hosni (red carrot) are the cultivated and edible varieties [2]. The wild subtypes of the carrot include the subspecies *carota*, *maritimus*, *major*, *maximus*, *gummiferi*, *hispanicus*, *commutatus*, *fontanesii*, and *bocconeii* [3]. Selective breeding of an ancestral wild form of carrots, *D. carota* ssp. *carota*, over the centuries led to the origination of a cultivated form of *D. carota*, later made to be known as *D. carota* ssp. *sativus* [4]. Most of the available literature focuses on this specific subspecies, and it has

been a topic of interest for researchers since the day of its cultivation. Differentiation among *D. carota* L. subspecies is difficult as the carrot is an outcrossing species, and both cultivated and wild carrot samples coexist, thus presenting the possibility of hybrids' formation. Nonetheless, Thellung classifies the subspecies into two groups, *eucarota* and *gummiferi*, each classified into five more subspecies. The *eucarota* group includes the subspecies *sativus*, *carota*, *maritimus*, *major*, and *maximus*; these plants are mostly annual or biennial. Plants of the *gummiferi* group, on the other hand, are perennials and include the subspecies *commutatus*, *hispanicus*, *fontanesii*, *bocconei*, and *gummifer* [3], among others.

Multiple studies have been conducted to investigate the chemical composition of the different *D. carota* subspecies, which revealed the abundance of terpenes, phenolics, and flavonoids [5–8]. The chemical composition of the plant extracts differed between multiple subspecies as well as among plants of the same subspecies. According to Maxia et al. [6], the geographical origin of the plant sample, the plant organs, as well as its stage of development may affect its chemical composition. However, it was generally noted that monoterpenes and/or sesquiterpenes are mostly present in leaves, stems, and blooming umbels [6,7,9].

Traditionally, the wild carrot was utilized by the ancient Greeks and Romans for various medicinal purposes. Dioscorides, a Greek physician and botanist who lived in the 1st century AD, documented the uses of the plant in his renowned work “De Materia Medica” [10]. The plant is known to possess antilithic, diuretic, carminative, antipyretic, analgesic, anti-inflammatory, antiseptic, and anti-diabetic properties, among others [5,11–13]. Over the past two decades, the potential medicinal benefits of *D. carota* became clear, as its antioxidant, anticancer, anti-inflammatory, gastroprotective, hepatoprotective, antibacterial, and antifungal activities have been confirmed in-vitro and in-vivo as well [2,14].

Many review articles have been published about cultivated edible carrots, but none has been conducted on wild carrot subspecies. Therefore, the present article aims to present a comprehensive overview concerning the traditional uses, the phytochemical composition, and the pharmacological properties of known wild *D. carota* subspecies. In addition, the potential therapeutic uses of the wild carrot will be evaluated on the basis of the available pharmacological findings.

## 2. Methodology

A thorough search was conducted on the phytochemical composition, folk medicine applications, and pharmacological properties of wild carrot (*Daucus carota*) subspecies. Various electronic databases were consulted such as, PubMed, MDPI, Google Scholar, Science Direct, Elsevier, Springer, Taylor and Francis, Scopus, ACS publications, Wiley On-line Library, and the Lebanese American University Libraries' online database. These databases collectively offer a comprehensive and extensive range of articles available in the literature on this plant. To perform the bibliographic research, specific keywords were employed such as the wild carrot, *D. carota* ssp. *Carota*, *D. carota* ssp. *Hispanicus*, *D. carota* ssp. *hispidus*, *D. carota* ssp. *gummifer*, *D. carota* ssp. *hispanicus*, *D. carota* ssp. *maximus*, *D. carota* ssp. *maritimus*, and *D. carota* ssp. *major*. The emphasis of this review encompasses phytochemical composition and pharmacological properties including antioxidant, anticancer, anti-inflammatory, antibacterial, and antifungal activities as well as other ethnopharmacological practices. The data pertinent to this study were collected through a comprehensive review of 100 scholarly articles, 25 books, 2 encyclopedias, 2 conference papers, 2 web pages, and 1 meeting report, spanning the years 1927 to 2023. The inclusion criteria in this study were based on the credibility of the sources from which the data were collected. Only data obtained from sources with a high level of credibility were included, emphasizing the importance of dependable and authoritative information to support the study's findings. The gathered information corresponds to research studies conducted across various global locations, spanning the United States, Europe, the Mediterranean region, North Africa, the Middle East, Western Asia, and China.

### 3. Plant Description and Distribution

The wild carrot, commonly known as Bird's Nest, Bishop's Lace, and Queen Anne's lace [15], is generally a biennial plant (around 1 m height) with distinctive clusters of tiny white flowers that bloom during warm seasons. Unlike the cultivated carrot, the wild carrot has a thin tap root, hairy stems, two- to three-pinnate leaves, pinnate bracts below a concave umbel, when fruiting, and spiny fruits [1]. Its flowering period spans from May to September [16].

It is important to mention that due to the similarities in their appearances, incidences of confusion have been observed between *D. carota* and poison Hemlock (*Conium maculatum*), which is infamously linked to the death of the Greek philosopher Socrates [17,18]. Thus, providers and consumers should be able to identify and distinguish between the two plants. For example, *D. carota* stems and leaves are hairy, whereas *Conium maculatum* stems are smooth and display purple blotching. Regarding the aerial parts, *D. carota* has a purple/red flower at the center of the umbel, absent in *Conium maculatum*, and the umbels in *D. carota* appear flatter, resembling an umbrella in *Conium maculatum*. Finally, only *D. carota* demonstrates three-pronged bracts at the base of the flower/umbel. Despite their similar fern-like leaves, extreme caution should be exercised when identifying them [19].

*D. carota* L., which is originally native to Europe and southwestern Asia, grows in a temperate environment, as it can be usually spotted in Europe (Germany, England, Austria, Poland, Greece, Sweden, and Hungary), Africa (Tunisia, Egypt, and Mauritius), America (Canada, Puerto Rico, and the USA), and Asia (Iraq, Iran, Jordan, and Lebanon) [20].

### 4. Ethnopharmacological Use of *Daucus carota*

About 300,000 species of higher plants have been identified worldwide, of which 17,810 species have been documented by The Royal Botanic Gardens, Kew, as medicinal plants (State of the World's Plants, 2016). According to the World Health Organization (WHO), eighty percent of Africans utilize different forms of herbal medicine [21,22]. The global annual market of these herbal products is estimated at USD 60 billion (WHO, 2002). In China, herbal medicine played a significant role in containing and treating the 2004 severe acute respiratory syndrome (SARS) epidemic [23]. Additionally, the Generally Recognized-as-Safe (GRAS) list, approved by the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) in the USA, includes many of the commercial-plant-derived essential oils [24]. Apart from being a key ingredient in traditional cuisine and a fragrance component, *D. carota*, or 'carrot', has been used since ancient times for its nutritional and medicinal properties. Ancient Greeks and Romans valued and mentioned the plant in various reputable writings, and the different parts of the carrot were popularly utilized as medicinal sources in the treatment of many diseases [25]. Although less common than the domesticated variant of the carrot, the wild carrot has been used in foods as a flavoring agent in baked goods, frozen dairy, and meat products, as well as in gelatins, soups, and relishes [26].

The lesser-known subspecies, wild carrots, are no less important subtypes and have been utilized for various indications. It was first described in the third century BCE by the ancient Roman Diphilus of Siphnos to possess diuretic properties and by Pliny the Elder for its aphrodisiac effects [27]. According to "The Encyclopedia of Medicinal Plants", the wild carrot is a very beneficial plant that possesses hepatoprotective, diuretic, and detoxifying properties [28]. An infusion of its leaves prevents kidney-stone formation, treats existing stones, combats cystitis, promotes the pituitary gland release of gonadotrophins, and treats parasitic infections [12,28]. It presents itself as an effective remedy for numerous digestive, kidney, bladder, and menstrual ailments, as well as dropsy, flatulence, and edema [5,11–13,29–32]. The Romans were known to use the wild carrot as a component for contraception, a potent emmenagogue and an inhibitor of implantation [33]. Additionally, the plant was known as the traditional "morning after" contraception through the stimulation of the uterus [12,33,34]. Moreover, Duke et al. reported that the wild carrot exhibits carminative, diuretic, emmenagogue, and anthelmintic properties [35]. The seed oil has



also been used in anti-wrinkle creams [36]. In European and Middle Eastern folk medicine, wild carrot oil is used as an antiseptic and anti-inflammatory therapy for prostatitis and cystitis [5,11–13], diabetes mellitus, and gastric ulcers, as well as for myorelaxation [37].

Wild carrot is consumed in salads as part of the Mediterranean diet and is used as a food additive in some recipes [38]. People eat its young taproot cooked, consume its flower umbels after French frying, and use its seed oil as a flavoring agent in beverages and food products [39]. In Lebanese folk medicine, the plant is used for protection against hepatic diseases and the treatment of diabetes, gastric ulcers, muscle pain, and cancer. Professor Nehme, in his book the ‘Wild Flowers of Lebanon’, mentioned that the aromatic seeds of the wild carrot were used as a vermifuge, a diuretic, an antidote for snake bites, and for sterility [40]. Traditional medicine presents *D. carota*, whether cultivated or wild, as a highly beneficial plant with promising potential for treating various ailments. The traditional uses of the wild carrot in various ethnic groups are presented in Table 1.

**Table 1.** Traditional uses of the wild carrot in various ethnic groups.

| Ethnic Groups    | Traditional Uses                                                                                                                                                                                                                                                                                                          | References    |
|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| Native Americans | Used for various medicinal purposes, including as a diuretic and for digestive issues. Wild carrot seeds are included in a recipe specifically for female contraception.                                                                                                                                                  | [34,41,42]    |
| Romans           | Used as a component for contraception and contragestion, as well as for its diuretic properties and aphrodisiac effects.                                                                                                                                                                                                  | [12,27,33,34] |
| Greeks           | Seeds are recommended for their anti-fertility properties and their role as emmenagogues.                                                                                                                                                                                                                                 | [33,34]       |
| Persians         | Leaf and root are recognized as diuretic, beneficial for fertility, and efficacious against conditions such as a cough, pleurisy, corrosive ulcers, and dropsy. Additionally, the seeds are noted for their diuretic and emmenagogic properties and is considered deobstruent and useful for alleviating intestinal pain. | [43]          |
| Indians          | Used in Ayurvedic medicine for various purposes, including as a diuretic and to treat digestive issues. The seeds are used as a method of controlling women’s fertility and the induction of abortion.                                                                                                                    | [44–47]       |
| Turkish          | Seeds employed in Turkish folk medicine for the treatment of gastrointestinal and respiratory disorders.                                                                                                                                                                                                                  | [48]          |
| Chinese          | Fruits utilized for the management of ascariasis, enterobiasis, and tapeworm disease. The roots and basal leaves are employed to strengthen the spleen and treat conditions such as dyspepsia and chronic dysentery.                                                                                                      | [49,50]       |
| Europeans        | Wild carrot essential oil is used as an antiseptic for the treatment of cystitis and prostatitis as well to treat urinary calculus, gout, and lithuria.                                                                                                                                                                   | [11–13]       |
| Lebanese         | Employed in the protection against hepatic diseases and in the treatment of diabetes, gastric ulcers, muscle pain, and cancer. Also, it is utilized as a vermifuge, a diuretic, an antidote for snake bites, and for managing sterility.                                                                                  | [40]          |

## 5. Phytochemistry and Bioactive Compounds/Chemical Composition

To better understand and characterize the medicinal benefits of the wild carrot, a phytochemical investigation is warranted. Numerous scholars have conducted extensive studies on the different *D. carota* subspecies to identify and characterize their bioactive constituents. As illustrated in Table 2, terpenoids and phenolics emerge as two significant chemical classes identified, with the terpenoids further classified into monoterpenes (e.g.,  $\alpha$ -pinene and geranyl acetate), sesquiterpenes (e.g., humulene and carotol), diterpenes (e.g., phytol), triterpenes (e.g., squalene), and tetraterpenes (e.g.,  $\alpha$ -carotene). Phenolics encompass phenylpropanoids, flavonoids, and tannins.



**Table 2.** Classification of different classes of chemical compounds.

| Class      | Types            | Examples                                                                                                                                                 |
|------------|------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|
| Terpenoids | Monoterpenes     | Geraniol, Limonene, $\alpha$ -Pinene, $\beta$ -Pinene, Sabinene, $\alpha$ -Terpinene, $\beta$ -Myrcene, Geranyl acetate, Linalool, and $\alpha$ -Thujone |
|            | Sesquiterpenes   | Bergamotene, Humulene, Nerolidol, Selinene, Farnesol, Germacrene, Carotol, Caryophyllene, $\beta$ -Himachalene, and $\beta$ -Bisabolene                  |
|            | Diterpenes       | Phytol, Vitamin A <sub>1</sub> , and Cembrene                                                                                                            |
|            | Triterpenes      | Squaline, Saponins, and Ginsenoide                                                                                                                       |
|            | Tetraterpenes    | Carotenoids (e.g., $\alpha$ -carotene, $\beta$ -carotene, lycopene) and Xanthophylls (lutein)                                                            |
| Phenolics  | Phenylpropanoids | Apigenin, Quercetin, Myristicin, and Methylisoeugenol                                                                                                    |
|            | Flavonoids       | Luteolin, Apigenin, Quercetin, Myristicin, and Kaempferol                                                                                                |
|            | Tannins          | Gallic acid and Ellagic acid                                                                                                                             |

As is evident in Table 3, *D. carota* subspecies have proven to be rich in various chemical components, with some being familiar and well-studied while others are new and worth further attention. In particular, the monoterpenes  $\alpha$ -pinene, geranyl acetate, and sabinene, along with the sesquiterpene carotol, stand out as the most prominent constituents in different *D. carota* subspecies, exhibiting variable percentages. These compounds are noteworthy for their diverse pharmacological properties, including anti-inflammatory, antibacterial, and antifungal activities [51–63]. Although the country of origin and the plant organ differ, most of the components are shared among all subspecies, with some exceptions that are clearly evident. For example,  $\beta$ -2-himachalen-6-ol is a novel sesquiterpene that was extracted, identified, and reported for the first time by a Lebanese team of researchers while studying the Lebanese variant of *D. carota* ssp. *carota*. This compound is exclusive to this subspecies grown in Lebanon, as it was never reported in any other country while studying the same variant. Furthermore, the two phytochemicals, apiole and myristicin are unique to the Algerian *D. carota* ssp. *hispanicus*. Table 3 summarizes the different *D. carota* subspecies and their main chemical components.

**Table 3.** Summary of the main chemical components of different *Daucus carota* subspecies from different countries of origin (percentages > 3% were reported).

| <i>Daucus carota</i> ssp. | Plant Organ            | Country  | Main Components                                                                                                                                                                                                                                                                                                                                                                                   | References |
|---------------------------|------------------------|----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>carota</i>             | Umbels                 | Lebanon  | $\beta$ -2-himachalen-6-ol (33%), $\alpha$ -longipinene (3.22–15.87%), methyl linoleate (8.26%), ( <i>E</i> )-methylisoeugenol (2.21–7.92%), 2-butanone (5.95%), $\alpha$ -Selinene (4.53–5.69%), Elemicin (4.03–4.93%), $\beta$ -Asarone (4.07%), $\beta$ Himachalene (2.24–4.63%), n-hexadecanoic acid (3.72%), humulene (3.27%), himachala-1,4-diene (3.09%), $\beta$ -Bisabolene (1.76–3.78%) | [37,64,65] |
|                           | Flowering umbels       | Portugal | $\alpha$ -Pinene (37.9%), geranyl acetate (15%), ( <i>E</i> )-caryophyllene (4.9%), $\beta$ -Pinene (3.5%),                                                                                                                                                                                                                                                                                       | [6]        |
|                           | Umbels with ripe seeds |          | Geranyl acetate (65%), $\alpha$ -Pinene (13%)                                                                                                                                                                                                                                                                                                                                                     |            |
|                           | Flowering umbels       | Italy    | Carotol (25.1%), 11 $\alpha$ H-himachal-4-en-1- $\beta$ -ol (21.6%), $\beta$ -bisabolene (17.6%), elemicin (6.4%)                                                                                                                                                                                                                                                                                 |            |
|                           | Umbels with ripe seeds |          | $\beta$ -bisabolene (51%), ( <i>E</i> )-methyl isoeugenol (10%), 11 $\alpha$ H-himachal-4-en-1- $\beta$ -ol (9%), elemicin (5.2%), $\alpha$ -longipinene (3.1%)                                                                                                                                                                                                                                   |            |

Table 3. Cont.

| <i>Daucus carota</i> ssp. | Plant Organ                   | Country  | Main Components                                                                                                                                                                                                                                                                                  | References |
|---------------------------|-------------------------------|----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>carota</i>             | Ripe umbels                   | Portugal | Geranyl acetate (29%), $\alpha$ -Pinene (27.2%), Limonene (9%), 11 $\alpha$ H-Himachal-4-en-1- $\beta$ -ol (9.2%), Carotol (6.2%), $\beta$ -Pinene (4.5%)                                                                                                                                        | [66]       |
|                           | Ripe umbels with mature seeds | Tunisia  | Carotol (3.5–55.7%), Elemecin (1.4–35.3%), 11 $\alpha$ H-Himachal-4-en-1- $\beta$ -ol (12.7–17.4%), Sabinene (12–14.5%), $\alpha$ -Selinene (7.4–8.6%), Eudesm-7(11)-en-4-ol (8.2–8.5%), $\beta$ -Bisabolene (5.5–7.6%), (Z)- $\beta$ -Farnesene (1.6–5%), (E)- $\alpha$ -Bergamotene (0.2–3.8%) | [9]        |
|                           | Herbs                         | Poland   | Sabinene (30.1%), $\alpha$ -Pinene (30%), Terpinen-4-ol (6.1%), limonene (5.3%), myrcene (5.2%)                                                                                                                                                                                                  | [8]        |
|                           | Flowering Umbels              |          | $\alpha$ -Pinene (42%), Sabinene (19.5%), limonene (3.7%), myrcene (3.1%)                                                                                                                                                                                                                        |            |
|                           | Mature Umbels                 |          | Sabinene (40.5%), $\alpha$ -Pinene (17.2%), geranyl acetate (16.5%), Terpinen-4-ol (4.9%),                                                                                                                                                                                                       |            |
|                           | Aerial parts                  | France   | (E)-methyloisoeugenol (21.8–33%), $\beta$ -Bisabolene (4.4–21.3%), Elemicin (11.4–16.3%), $\alpha$ -Pinene (15.9–24.9%), Sabinene (2.7–3.7%), Myrcene (2–3.5%), $\alpha$ -Terpinen-4-ol (0.5–3.5%)                                                                                               | [5,7]      |
|                           | Flowers                       | Algeria  | $\alpha$ -Pinene (10.9%), $\alpha$ -Asarone (9.8%), $\beta$ -Bisabolene (7.6%), $\beta$ -Caryophyllene (7.1%), Sabinene (7%), Daucol (3.2%), Limonene (3%)                                                                                                                                       | [67]       |
|                           | Leaves + Stems                |          | $\alpha$ -Pinene (10.6%), $\alpha$ -Asarone (9.4%), $\beta$ -Bisabolene (9.3%), Sabinene (7.2%), Carotol (6.8%), E- $\alpha$ -Bisabolene (6.3%), Daucol (5.3%), $\beta$ -Caryophyllene (4.3%), Limonene (4%)                                                                                     |            |
|                           | Aerial parts                  |          | $\alpha$ -Pinene (21.3%), $\alpha$ -Asarone (18.4%), $\beta$ -Bisabolene (7.3%), Sabinene (6.5%), Limonene (6.4%), Carotol (3.5%), Terpinen-4-ol (3.5%), $\beta$ -Caryophyllene (3.3%), E- $\alpha$ -Bisabolene (3.2%)                                                                           |            |
|                           | Leaves                        |          | $\alpha$ -Pinene (27.44%), sabinene (25.34%), Germacrene D (16.33%)                                                                                                                                                                                                                              |            |
|                           | Seeds                         |          | Geranyl acetate (52.45%), Cedrone S (14.04%), Asarone (11.39%), $\beta$ -bisobolene (4.83%), Ar-himachalene (3.54%)                                                                                                                                                                              |            |
|                           | Ripe fruits                   |          | Sabinene (27.16%), $\alpha$ -pinene (21.3%), $\alpha$ -muurolene (8.23%), $\beta$ -caryophyllene (6.82%), $\alpha$ -ylangene (5.21%), $\beta$ -Pinene (3.9%)                                                                                                                                     |            |
|                           | Unripe fruits                 |          | $\alpha$ -muurolene (10.97%), sabinene (10.67%), caryophyllene oxide (7.7%), $\alpha$ -amorphene (7.57%), $\alpha$ -pinene (7.05%), carotol (6.15%), dimenone (5.28%), $\alpha$ -ylangene (4.88%),                                                                                               |            |
|                           | Flowers                       | Serbia   | $\alpha$ -Pinene (51.23%), Limonene (9.59%), Sabinene (8.62%), $\beta$ -Myrcene (7.18%), Terpinen-4-ol (3.48%), $\beta$ -Pinene (3.35%)                                                                                                                                                          | [69]       |
|                           | Roots                         |          | Sabinene (36.39%), $\alpha$ -Pinene (24.56%), Limonene (6.53%), $\beta$ -Pinene (5.39%)                                                                                                                                                                                                          |            |
|                           | Leaves                        |          | $\alpha$ -Pinene (30.83%), Limonene (8.6%), $\beta$ -Myrcene (5.6%), Germacrene D (4.56%)                                                                                                                                                                                                        |            |
|                           | Stems                         |          | $\alpha$ -Pinene (18.53%), $\alpha$ -Bisabolol (6.02%), Limonene (5.74%), $\beta$ -Myrcene (3.4%), Sabinene (3.23%)                                                                                                                                                                              |            |
|                           | Fruits                        | Portugal | Geranyl acetate (28.7–65%), $\alpha$ -Pinene (13–27.1%), 11 $\alpha$ H-Himachal-4-en-1- $\beta$ -ol (0.5–9.4%), Limonene (1.2–9%), $\beta$ -Pinene (2.3–4.5%)                                                                                                                                    | [70]       |

Table 3. Cont.

| <i>Daucus carota</i> ssp. | Plant Organ            | Country       | Main Components                                                                                                                                                                                                                                   | References |
|---------------------------|------------------------|---------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>carota</i>             | Roots                  | Vienna        | $\alpha$ -Terpinolene (26.2–56.3%), $\beta$ -Pinene (4.1–8.2%), <i>p</i> -Cymene (2.7–7.4%), Sabinene (5.6–5.9%), $\gamma$ -terpinene (0.9–5.6%), Limonene (5.5%), Myristicin (4.9–5.1%)                                                          | [1]        |
|                           | Leaves                 |               | $\alpha$ -Pinene (20.9–44.8%), Sabinene (11.3–19.5%), Germacrene D (4.9–14%), Limonene (3.9–12.7%), Myrcene (4–11.2%), $\beta$ -Pinene (1.3–5.9%), Caryophyllene (1.2–3.7%)                                                                       |            |
|                           | Fruits                 |               | Sabinene (21.5–46.6%), $\alpha$ -Pinene (23.5–30.4%), Geranyl acetate (3.9–28.1%), $\beta$ -Pinene (3–13.1%), $\alpha$ -Thujene (1–8.8%), $\gamma$ -terpinene (0.3–4.1%), Myrcene (3.4–3.9%)                                                      |            |
|                           | Seeds                  | Lithuania     | Sabinene (28.2–37.5%), $\alpha$ -Pinene (16–24.5%), Terpinen-4-ol (5–6%), $\gamma$ -terpinene (2.9–6%), Limonene (3–4.2%)                                                                                                                         | [61]       |
|                           | Leaves                 | Uzbekistan    | Carotol (68.3%), Daucene (5%), <i>trans</i> - $\beta$ -Farnesene (3.7%), $\beta$ -Bisabolene (3.3%), $\alpha$ -Pinene (3.1%)                                                                                                                      | [71]       |
|                           | Flowers                |               | Carotol (68.8%), Daucene (4.7%), Daucol (3.4%), <i>trans</i> - $\beta$ -Farnesene (3.3%)                                                                                                                                                          |            |
|                           | Petals                 |               | Carotol (78.3%)                                                                                                                                                                                                                                   |            |
|                           | Fruits                 |               | Carotol (69.8%), Daucene (9%), <i>trans</i> - $\alpha$ -Bergamotene (4.7%), <i>trans</i> - $\beta$ -Farnesene (3.7%)                                                                                                                              |            |
|                           | Umbels                 | United States | $\alpha$ -Pinene (33.02%), $\beta$ -Pinene (25.77%), Borneol (10.4%), Myrcene (6.41%), Limonene (5.34%), $\gamma$ -terpinene (4.97%)                                                                                                              | [72]       |
| <i>maximus</i>            | Ripe and mature fruits | Egypt         | ( <i>E</i> )-methylisoeugenol (37.22%), $\beta$ -bisabolene (34.7%), $\beta$ -Asarone (17.65%)                                                                                                                                                    | [2]        |
|                           | Leaf                   |               | Preisocalamendiol (17.95%), Shyobunone (16.84%), $\beta$ -Cubebene (12.72%), Tridecane (3.411%), Linalool (3.34%), ( <i>E</i> )-2-Nonenal (3.22%)                                                                                                 |            |
|                           | Stem                   |               | Preisocalamendiol (32.69%), Shyobunone (24.33%), $\alpha$ -Pinene (4.37%), $\beta$ -Cubebene (3.55%)                                                                                                                                              |            |
|                           | Fruits                 | Portugal      | $\alpha$ -Pinene (10–25.9%), $\alpha$ -Asarone (5.8–25.8%), Geranyl acetate (3.4–16%), $\beta$ -bisabolene (8.3–15.1%), ( <i>E</i> )-methylisoeugenol (8.2–15.7%), Elemicin (4.9–13.6%), $\beta$ -Pinene (4–6.8%), Limonene (1.8–3.3%) [73]       | [70]       |
|                           | Ripe umbels            | Portugal      | $\alpha$ -Pinene (22.2%), Geranyl acetate (16%), $\beta$ -bisabolene (11.5%), $\alpha$ -asarone (9.8%), ( <i>E</i> )-methylisoeugenol (8%), Elemicin (6%), $\beta$ -Pinene (5.8%)                                                                 | [74]       |
|                           | Green seeds            | Italy         | Carotol (44.68%), $\beta$ -bisabolene (12.72%), Isoelemicin (11.51%), Geranyl acetate (4.36%)                                                                                                                                                     | [75]       |
| <i>maritimus</i>          | Flowers                | Tunisia       | Sabinene (51.6%), Terpinen-4-ol (11%), <i>p</i> -Cymene (4.2%), Eudesm-6-en-4 $\alpha$ -ol (3.6%)                                                                                                                                                 | [76]       |
|                           | Roots                  |               | Dillapiole (46.6%), Myristicin (29.7%), Limonene (3.6%)                                                                                                                                                                                           |            |
| <i>major</i>              | Flowers                | Italy         | $\alpha$ -Pinene (24.4%), Sabinene (13.3%), Geranyl acetate (13%), <i>epi</i> - $\alpha$ -Cadinol (8.5%), Myrcene (4.8%), $\beta$ -Oplophenone (4.3%)                                                                                             | [62]       |
|                           | Fruits                 |               | Geranyl acetate (34.2%), $\alpha$ -Pinene (12.9%), Geraniol (6.9%), Myrcene (4.7%), <i>epi</i> - $\alpha$ -Bisabolol (4.5%), Sabinene (3.3%)                                                                                                      |            |
| <i>halophilus</i>         | Flowering Umbels       | Portugal      | Sabinene (28.3–33.8%), $\alpha$ -Pinene (12.6–16%), Limonene (11–11.8%), ( <i>E</i> )-methylisoeugenol (0.7–7.4%), Elemicin (5.9–6.2%), $\beta$ -Bisabolene (0.4–5.3%), Terpinene-4-ol (4.1–4.8%), Myrcene (3.2–4.7%), $\beta$ -Pinene (2.3–5.1%) | [73]       |
|                           | Ripe Umbels            |               | Elemicin (26–31%), Sabinene (27.6–29%), $\alpha$ -Pinene (10.1–12.2%), Limonene (5.5–6.5%), ( <i>E</i> )-methylisoeugenol (0.5–6.9%)                                                                                                              |            |
|                           | Fruits                 |               | Elemicin (15–31%), Sabinene (9–29%), $\alpha$ -Pinene (12.2–23%), Limonene (5.5–12%), ( <i>E</i> )-methylisoeugenol (0.5–7.4%), Terpinen-4-ol (2–4.7%)                                                                                            | [70]       |

Table 3. Cont.

| <i>Daucus carota</i> ssp. | Plant Organ  | Country  | Main Components                                                                                                                                                                          | References |
|---------------------------|--------------|----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>hispanicus</i>         | Roots        | Algeria  | Apiole (80.3%), Myristicin (16.6%)                                                                                                                                                       | [77]       |
|                           | Aerial parts |          | Myristicin (73.2%), Epiglobulol (5.1%), Germacrene D (3.1%)                                                                                                                              |            |
|                           | Stems        |          | Myristicin (66.9%), $\alpha$ -Thujene (4.3%)                                                                                                                                             |            |
|                           | Leaves       |          | Myristicin (80.2%), Epiglobulol (3.1%)                                                                                                                                                   |            |
|                           | Flowers      |          | Myristicin (83.8%), Germacrene D (6.4%)                                                                                                                                                  |            |
| <i>gummifer</i>           | Fruits       | Spain    | Geranyl acetate (51.74–76.95%), Sabinene (4.42–11.13%), Terpinen-4-ol (0.93–8.17%), Linalool (3.97–5.18%) [78]                                                                           | [78]       |
|                           |              | Portugal | Geranyl acetate (18–55%), $\alpha$ -Pinene (11–31%), Carotol (5–15%), Sabinene (2.1–10%), Limonene (5.8–9%), Germacrene D (2–5.5%), $\beta$ -Pinene (3.8–5.2%), Myrcene (2.1–3.7%)       | [73]       |
|                           | Ripe umbels  | Portugal | Geranyl acetate (37%), Limonene (5.8%), $\alpha$ -Pinene (30.9%), $\beta$ -Pinene (3.8%)                                                                                                 | [24]       |
| <i>hispidus</i>           | Aerial parts | Tunisia  | (4R)-1- <i>p</i> -menthen-6,8-diol, 1- <i>p</i> -menthen-4,7-diol, (1R,2R,4R)- <i>p</i> -menthane-1,2,4-triol, $\beta$ -sitosterol 3- <i>O</i> -glucoside (abundance not specified) [79] | [79]       |

## 6. Pharmacological Activities

The popular use of *D. carota* in traditional medicine has attracted the attention of many researchers to investigate the claimed benefits. Consequently, numerous studies have been carried out to investigate the proposed pharmacological properties. Relying on its ethnopharmacological indications, mainly to treat cancer, inflammation, and infections, both in vitro and, to a lesser extent, in vivo models were implemented to assess its pharmacological relevance. The findings of these studies are discussed in the sections below.

### 6.1. Anticancer Activity

With the increasing need for optimizing cancer therapy, extensive studies were conducted to assess the potential benefit of herbal options in oncology. Regarding the anticancer potential of wild *D. carota*, only *D. carota* ssp. *carota* was studied and demonstrated to possess these properties. Zeinab et al. [65] assessed the chemoprotective effects of Lebanese *Daucus carota* ssp. *carota* dried umbels acetone-methanol (50:50) oil extract on 7,12-dimethyl benz(a)anthracene (DMBA)-initiation-12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-induced skin carcinogenesis in mice. The extract was administered through topical applications, gavage, and intraperitoneal (IP) injections. Both intraperitoneal and topical treatments displayed a significant inhibition of papilloma number and volume, accompanied by an improved tissue histology.

Moreover, recent findings revealed that *D. carota* oil extract (DCOE) demonstrated substantial preventive and therapeutic effects against DMBA-induced breast cancer in rats. Animals pre-treated with DCOE exhibited prolonged survival and reduced tumor incidence. Also, DCOE treatment post-tumor induction resulted in a significant inhibition of tumor volume [80]. Shebaby et al. [37] reported that DCOE treatment exhibited important cytotoxicity against several human cancer cell lines (colon: HT-29 and Caco-2; breast: MCF-7 and MDA-MB-231). According to the authors, the anticancer activity of DCOE may be attributed to the presence of major sesquiterpenes including  $\beta$ -caryophyllene, caryophyllene oxide,  $\alpha$ -humulene, and a prominent compound C<sub>15</sub>H<sub>26</sub>O (later identified as  $\beta$ -2-himachalen-6-ol), in addition to other minor constituents acting in synergy. Similarly, Ambrož et al. [81] reported that  $\beta$ -caryophyllene and its oxide, as well as  $\alpha$ -humulene, obtained from *Myrica rubra* leaves, exhibited significant anticancer properties in CaCo-2 cells and other intestinal cancer cell lines, synergistically potentiating the efficacy of doxorubicin cytotoxicity. Additionally, in 2007, Legault and Pichette [82] showed that treatment with  $\beta$ -caryophyllene caused a significant enhancement of the anticancer ef-

fects of isocaryophyllene,  $\alpha$ -humulene, and paclitaxel on MCF-7 cells. Further studies also confirmed the cytotoxic activity of these highlighted compounds [83–87]. In 2019, Hammami et al. [79] reported that while (4R)-1-*p*-menthen-6,8-diol, a polyol menthane monoterpene, isolated from *D. carota* ssp. *hispidus*, did not induce cytotoxicity against human mouth squamous carcinoma (HSC-2) and human cervical (HeLa) cancer cells, while (1R,2R,4R)-*p*-menthane-1,2,4-triol and (4R)-1-*p*-menthen-4,7-diol (2) exhibited weak activity against the same cell lines.

Furthermore, in 2015, Tawil et al. [25] reported that DCOE induced caspase-dependent apoptotic cell death in human Acute Myeloid Leukemia (AML) cells, partially through the Mitogen-Activated Protein Kinase (MAPK)-dependent mechanism. These kinases regulate important cellular mechanisms such as proliferation, stress responses, immune defense, and apoptosis [88]. DCOE was eluted into four fractions (pentane:diethyl ether, 50:50 (F2), diethyl ether, 100%) and F4 (chloroform:methanol, 93:7) and tested against various human cell lines [89]. The pentane (100%) and pentane/diethyl ether (50:50) fractions displayed the highest anticancer activity against MDA-MB-231, MCF-7, HT-29, and Caco-2 cells. Inhibition of cell proliferation in MDA-MB-231 and HT-29 cells was attributed to cell cycle arrest and apoptosis. The latter was mediated via the suppression of the MAPK/Erk pathway in MDA-MB-231 cells and the PI3K/Akt and MAPK/Erk pathways in HT-29 cells [89,90]. In another study, the four DCOE fractions exhibited significant in vitro cytotoxic effects against both tumorigenic and non-tumorigenic human epidermal keratinocytes (HaCaT cells and HaCaT-ras variants). The anti-tumor effect of the pentane:diethyl ether (50:50) fraction was also observed in a DMBA/TPA skin carcinogenesis mouse model, where a significant decrease in papilloma incidence, yield, and volume was noted at weeks 15, 18, and 21. This activity was attributed to its major compound  $\beta$ -2-himachalene-6-ol [91]. Furthermore, the same fraction displayed a significant decrease in cell motility and invasion as well as an increase in cell adhesion in lung (A549), skin melanoma (B16F-10), breast (MDA-MB231), and SF-268 (glioblastoma) cells. These effects are partially due to the inhibition of *p*-GTPases, Rac, and CDC42 [92]. Later on,  $\beta$ -2-himachalene-6-ol was reported to cause significant cytotoxicity against various human cancer cell lines by inducing apoptosis and inhibiting the PI3K/Akt and the MAPK/Erk pathways. Moreover,  $\beta$ -2-Himachalene-6-ol decreased 2D cell motility and 3D invasion and increased cell adhesion in SF-268 [64]. Flow cytometry assays demonstrated that the treatment of HaCaT-ras II-4 with  $\beta$ -2-himachalene-6-ol caused a cell cycle arrest, an up-regulation of the p21 protein, and induced apoptosis through increasing the levels of cleaved caspase-3 and pro-apoptotic BAX proteins and by decreasing in the anti-apoptotic Bcl-2 protein [93,94]. Furthermore,  $\beta$ -2-himachalene-6-ol displayed significant chemo-preventive and chemo-therapeutic effects in the DMBA/TPA skin cancer mouse model by reducing papilloma incidence, yield, and volume. This effect was partially attributed to promoting the apoptosis and inhibition of the MAPK/ERK and PI3K/AKT pathways, without inducing significant toxicity [93,95]. Additional in vitro studies showed that  $\beta$ -2-himachalene-6-ol caused cell cycle arrests and induced apoptosis and DNA fragmentation in SW1116 colon cancer cells. A Western blot analysis showed that  $\beta$ -2-himachalene-6-ol upregulated the p53, p21, and BAX proteins while downregulating the Bcl-2, procaspase-3, and PARP proteins. In 2019, Daaboul et al. [96] revealed that  $\beta$ -2-himachalene-6-ol treatment caused a dose-dependent decrease in cytotoxicity, induced cell cycle arrests, and promoted apoptosis in murine breast cancer cell line 4T1, along with a reduction in the primary tumor size and an inhibition of metastasis in mice. Thus, the aforementioned literature provides enough evidence to support the claimed traditional anticancer use of *D. carota* ssp. *carota* in Lebanon. In conclusion, we believe that *D. carota* ssp. *carota* is worth further pre-clinical investigations to confirm its efficacy and safety. The above findings and Tables 4 and 5 summarize the in vitro and in vivo anticancer effects of *D. carota* ssp. *carota*.



**Table 4.** In vitro anticancer effects of Lebanese *D. carota* ssp. *carota*.

| Extracts/Compounds                                                                                                                                              | Detail                                                                | Concentration/Dose                                                | References |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|-------------------------------------------------------------------|------------|
| Methanol/Acetone (1:1)<br><i>Daucus carota</i> oil extract (DCOE)                                                                                               | Cytotoxicity against U937                                             | IC <sub>50</sub> = 1 µg/mL                                        | [25]       |
|                                                                                                                                                                 | Cytotoxicity against KG-1                                             | IC <sub>50</sub> = 1 µg/mL                                        |            |
|                                                                                                                                                                 | Cytotoxicity against HL60                                             | IC <sub>50</sub> = 5.5 µg/mL                                      |            |
|                                                                                                                                                                 | Cytotoxicity against TF1-VSrc                                         | IC <sub>50</sub> = 7.3 µg/mL                                      |            |
|                                                                                                                                                                 | Cytotoxicity against Mono-Mac-6                                       | IC <sub>50</sub> = 12.4 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against Mono-Mac-1                                       | IC <sub>50</sub> = 13.2 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against TF1-VRaf                                         | IC <sub>50</sub> = 14.4 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against MV-4-11                                          | IC <sub>50</sub> = 14.9 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against ML1                                              | IC <sub>50</sub> = 19.6 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against TF1-HaRas                                        | IC <sub>50</sub> = 26 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against ML2                                              | IC <sub>50</sub> = 26.2 µg/mL                                     | [37]       |
|                                                                                                                                                                 | Cytotoxicity against human peripheral blood mononuclear cells (PBMCs) | IC <sub>50</sub> > 100 µg/mL                                      |            |
|                                                                                                                                                                 | Cytotoxicity against HT-29                                            | IC <sub>50</sub> = 34 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against MDA-MB231                                        | IC <sub>50</sub> = 27 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against MCF-7                                            | IC <sub>50</sub> = 33 µg/mL                                       | [89]       |
|                                                                                                                                                                 | Cytotoxicity against Caco-2                                           | IC <sub>50</sub> = 30 µg/mL                                       |            |
| Pentane (100%) fraction (F1)<br>Pentane: diethyl ether (50:50) fraction (F2)<br>Diethyl ether (100%) fraction (F3)<br>Chloroform: methanol (93:7) fraction (F4) | F1: Cytotoxicity against MDA-MB-231                                   | IC <sub>50</sub> = 17 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against MCF-7                                            | IC <sub>50</sub> = 22 µg/mL                                       |            |
|                                                                                                                                                                 | F2: Cytotoxicity against MDA-MB-231                                   | IC <sub>50</sub> = 11 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against MCF-7                                            | IC <sub>50</sub> = 32 µg/mL                                       |            |
|                                                                                                                                                                 | F3: Cytotoxicity against MDA-MB-231                                   | IC <sub>50</sub> = 27 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against MCF-7                                            | IC <sub>50</sub> = 48 µg/mL                                       |            |
|                                                                                                                                                                 | F4: Cytotoxicity against MDA-MB-231                                   | IC <sub>50</sub> = 23 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against MCF-7                                            | IC <sub>50</sub> = 43 µg/mL                                       |            |
|                                                                                                                                                                 | F1: Cytotoxicity against HaCaT-ras A5                                 | IC <sub>50</sub> = 10.6 µg/mL                                     | [91]       |
|                                                                                                                                                                 | Cytotoxicity against HaCaT-ras II4                                    | IC <sub>50</sub> = 10.2 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against HaCaT                                            | IC <sub>50</sub> = 29.8 µg/mL                                     |            |
|                                                                                                                                                                 | F2: Cytotoxicity against HaCaT-ras A5                                 | IC <sub>50</sub> = 14.6 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against HaCaT-ras II4                                    | IC <sub>50</sub> = 11.4 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against HaCaT                                            | IC <sub>50</sub> = 33.2 µg/mL                                     |            |
|                                                                                                                                                                 | F3: Cytotoxicity against HaCaT-ras A5                                 | IC <sub>50</sub> = 19.1 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against HaCaT-ras II4                                    | IC <sub>50</sub> = 17.8 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against HaCaT                                            | IC <sub>50</sub> = 47.3 µg/mL                                     |            |
|                                                                                                                                                                 | F4: Cytotoxicity against HaCaT-ras A5                                 | IC <sub>50</sub> = 16.2 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against HaCaT-ras II4                                    | IC <sub>50</sub> = 14.5 µg/mL                                     | [90]       |
|                                                                                                                                                                 | Cytotoxicity against HaCaT                                            | IC <sub>50</sub> = 43.4 µg/mL                                     |            |
| Pentane (100%) fraction (F1)<br>Pentane: diethyl ether (50:50) fraction (F2)                                                                                    | F1: Cytotoxicity against HT-29                                        | IC <sub>50</sub> = 22 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against Caco-2                                           | IC <sub>50</sub> = 18.5 µg/mL                                     |            |
|                                                                                                                                                                 | F2: Cytotoxicity against HT-29                                        | IC <sub>50</sub> = 17.5 µg/mL                                     |            |
|                                                                                                                                                                 | Cytotoxicity against Caco-2                                           | IC <sub>50</sub> = 19 µg/mL                                       | [64]       |
| β-2-himachalen-6-ol (HC)                                                                                                                                        | Cytotoxicity against B16F-10                                          | IC <sub>50</sub> = 13 µg/mL                                       |            |
|                                                                                                                                                                 | Cytotoxicity against Caco2                                            | IC <sub>50</sub> = 8 µg/mL                                        |            |
|                                                                                                                                                                 | Cytotoxicity against MB-MDA-231                                       | IC <sub>50</sub> = 6 µg/mL                                        |            |
|                                                                                                                                                                 | Cytotoxicity against A549                                             | IC <sub>50</sub> = 5 µg/mL                                        |            |
|                                                                                                                                                                 | Cytotoxicity against SF-268                                           | IC <sub>50</sub> = 4 µg/mL                                        |            |
|                                                                                                                                                                 | Cytotoxicity against SW116                                            | IC <sub>50</sub> = 18 and 14.5 µg/mL,<br>24 and 48 h respectively | [94]       |
|                                                                                                                                                                 | Cytotoxicity against HaCaT-ras II-4                                   | IC <sub>50</sub> = 7 µg/mL                                        | [93]       |
|                                                                                                                                                                 | Cytotoxicity against HaCaT-ras II-4                                   | IC <sub>50</sub> = 8 µg/mL                                        | [95]       |
|                                                                                                                                                                 | Cytotoxicity against 4T1                                              | IC <sub>50</sub> = 7 µg/mL                                        | [96]       |

**Table 5.** In vivo anticancer effects of Lebanese *D. carota* ssp. *carota*.

| Cancer Model                                       | Extracts/Compounds                                                | Mode of Treatment<br>(Dose and Frequency of Administration)                                                                                                                                                         | Results                                                                                                                                                                                                        | References |
|----------------------------------------------------|-------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| DMBA/TPA-induced skin carcinogenesis model in mice | Methanol/Acetone (1:1)<br><i>Daucus carota</i> oil extract (DCOE) | Gavage, 20 µL of 100% oil<br>Intraperitoneal, 0.3 mL of 2% oil.<br>Topical, 0.2 mL of 100% oil; 50% oil; 5% oil.                                                                                                    | Minimal effects seen with gavage administration. Significant decrease in tumor volume, delay in tumor appearance, and inhibition of tumor incidence and yield with intraperitoneal and topical administration. | [65]       |
|                                                    |                                                                   | Chemoprevention:<br>Pre-treatment with DCOE (25 mg/kg) a week prior to cancer induction<br>Treatment twice weekly for 14 weeks.<br>Chemotherapeutic:<br>DCOE (25 mg/kg body weight; IP; thrice a week for 8 weeks). | Reduced tumor incidence. Protection against DMBA-induced toxicity. Significant inhibition of tumor volume. No decrease in body weight as compared to cisplatin.                                                | [80]       |
|                                                    | Pentane: diethyl ether (50:50) fraction (F2)                      | Intraperitoneal treatment (10–200 mg/kg)                                                                                                                                                                            | Significant inhibition of papilloma incidence, yield, and volume at weeks 15, 18, and 21.                                                                                                                      | [91]       |
|                                                    | β-2-himachalen-6-ol (HC)                                          | Topical (5%).<br>Intraperitoneal HC (25 mg/kg).                                                                                                                                                                     | Significant decrease in papilloma yield and volume at weeks 12, 16, and 18, and increase in survival rates. No decrease in weight with HC (safer).                                                             | [93]       |
|                                                    |                                                                   | Topical (5%).<br>Intraperitoneal HC (10, 25, and 50 mg/kg).                                                                                                                                                         | Significant decrease in papilloma yield, incidence, and volume, and twofold to threefold increase in survival rates.                                                                                           | [95]       |

### 6.2. Antibacterial Activity

With the increasing resistance to antibacterial agents and the need for alternatives to the current options, few studies have assessed the role of *D. carota* in eradicating bacterial infections. Only *D. carota* ssp. *carota*, *hispanicus*, *maritimus*, and *maximus* have been investigated and have been shown to possess antibacterial properties against various bacterial species. In 2005, Staniszewska et al. [8] tested the essential oil of *D. carota* ssp. *carota* umbels from Poland against four different species of bacteria, revealing a greater efficacy against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) than Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). Moreover, *C. jejuni* (reference strain and clinical isolates F38O11, LV7, and LV9) as well as other bacterial strains of the *Campylobacter* genus (*C. coli* and *C. lari*) were found to be equally susceptible to the antibacterial effect of the aerial parts of *D. carota* ssp. *carota* harvested in France [5]. Additionally, the oil was tested against a multidrug-resistant *C. jejuni* strain (99T403), and it exhibited comparable growth inhibition to that of non-resistant strains. The antibacterial effect was attributed to phenylpropanoids, elemicin, and (*E*)-methylisoeugenol. Ripe and unripe fruits, flowers, roots, leaves, and stems of *D. carota* ssp. *carota* oil extracts from Serbia were also investigated [69]. While the most remarkable activity was observed against *Bacillus*

*cereus*, the highest inhibition was attained by the ripe and unripe fruits and the lowest with the flower oil. The tested oils were more active than Streptomycin against *Escherichia coli* and *Pseudomonas aeruginosa*. Essential oils of *D. carota* ssp. *carota* ripe umbels from Portugal was also tested against Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*, and *Listeria monocytogenes*), and Gram-negative (*Salmonella typhimurium* and *Escherichia coli*) strains, revealing its higher effectiveness against Gram-positive bacteria [66]. The observed inhibition was attributed to the major compounds  $\alpha$ -pinene and geranyl acetate. Several studies have highlighted the antibacterial potential of  $\alpha$ -pinene against numerous bacterial species. Kovac et al. [38] reported that  $\alpha$ -pinene modulates *Campylobacter jejuni* antibiotic resistance by reducing the MIC value of triclosan, erythromycin, and ciprofloxacin by up to 512 times. This is achieved through targeting the antimicrobial efflux systems, enhancing microbial influx, reducing membrane integrity, and disrupting metabolisms [51]. Also,  $\alpha$ -pinene, isolated from Korean citrus species, demonstrated significant antibacterial activity against *Staphylococcus epidermidis* and *Propionibacterium acnes* [53]. Another study reported that  $\alpha$ -pinene demonstrated activity against the *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* strains [60]. The antibacterial activity of  $\alpha$ -pinene against various bacterial strains has been consistently reported in multiple studies [51–60,69].

Asilbekova et al. [71] reported that essential oils of different parts (leaves, flowers, petals, fruits) of *Daucus carota* ssp. *carota* from Uzbekistan demonstrated modest activity against *Staphylococcus aureus* and *Bacillus subtilis*. Furthermore, in 2009, Wehbe et al. [97] showed that the aqueous and methanolic extracts of Lebanese *D. carota* ssp. *carota* umbels exhibited a minimal inhibition of *Staphylococcus aureus* meti S and meti R (MIC<sub>20</sub> and 10 mg/mL, respectively), while no effect was observed against Gram-negative bacteria. Essential oils extracted from flowers and roots of Tunisian *D. carota* ssp. *maritimus* were also tested, showing broad-spectrum antibacterial activity against *P. aeruginosa*, *E. coli*, *E. faecalis*, *K. pneumoniae*, and *S. typhimurium* [76]. The flower-derived oil was more effective against *E. coli* (ESL $\beta$ ), while the root-derived oil demonstrated greater efficacy against *S. aureus*, *S. pneumoniae*, *Shigella* spp., and *E. faecalis*. The authors attributed these pharmacological activities to the significant presence of sabinene and terpinen-4-ol, as well as phenolic compounds like dillapiol and myristicin.

Sabinene has been reported to possess antibacterial activities against several Gram-positive and Gram-negative bacteria. The sabinene-type of the essential oil obtained from berries and leaves of *Juniperus excelsa* from Macedonia exhibited moderate activity against *Streptococcus pyogenes*, *Haemophilus influenzae*, *Campylobacter jejuni*, and *Escherichia coli* [98]. Moreover, sabinene, a major component of the *Zornia diphylla* essential oil (43.1%, whole plant), showed promising antibacterial activities, especially against in vitro *Salmonella typhi* [99].

Algerian *D. carota* ssp. *hispanicus* roots' and aerial parts' essential oils were shown to exhibit varied growth-inhibitory effects against Gram-positive and Gram-negative bacteria. The authors suggest that the antibacterial activity could be attributed to phenolic compounds such as myristicin and apiole [77]. Additionally, the seed oil obtained from the Italian *D. carota* ssp. *maximus* demonstrated significant inhibitory activity against most of the tested Gram-positive strains, particularly *Staphylococcus*, and only two Gram-negative pathogens, *Acinetobacter* and *Stenotrophomonas maltophilia* ICE272 [75]. Furthermore, the oil extract of Tunisian *D. carota* ssp. *carota* umbels displayed antibacterial activity against *E. coli* ATCC 35218 and *S. aureus* ATCC 43300 [9].

The above findings and Table 6 summarize the antibacterial activity of *D. carota* ssp. *carota*, *hispanicus*, *maritimus*, and *maximus*. The reported MIC values indicate weak to moderate antibacterial activity, which does not strongly support the claims of *D. carota* being an effective antiseptic and a remedy for prostatitis and cystitis in traditional medicine [5,11–13]. However, further in vitro and in vivo studies are recommended to confirm such claims.

**Table 6.** In vitro antibacterial effects of *D. carota* subspecies.

| DC Subspecies     | Plant Organ, Location                                             | Treatment                                                                    | Detail                                                                                                                                                                                                                                                                                                        | Concentration/MICs                                                                                                                                                           | References |
|-------------------|-------------------------------------------------------------------|------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>carota</i>     | Herbs and umbels, Poland                                          |                                                                              | Inhibitory effects against <i>Bacillus subtilis</i> and <i>Staphylococcus aureus</i> .<br>Inhibitory effects against <i>Escherichia coli</i> and <i>Pseudomonas aeruginosa</i> .                                                                                                                              | MIC = 3–5 µL/mL<br>MIC = ≥8 µL/mL                                                                                                                                            | [8]        |
|                   | Ripe and unripe fruits, flowers, roots, leaves, and stems; Serbia | Essential oil (hydrodistillation)                                            | All oils; strongest inhibitory effects against <i>Bacillus cereus</i> :<br>Ripe fruit oil;<br>Unripe fruit oil;<br>Flower oil;<br>Root oil;<br>Stem oil;<br>Leaf oil.                                                                                                                                         | MIC = 5.0–50.0 µL/mL<br>MIC = 5.0–25.0 µL/mL<br>MIC = 10.0–25.0 µL/mL<br>MIC = 50.0–400.0 µL/mL<br>MIC = 5.0–150.0 µL/mL<br>MIC = 25.0–250.0 µL/mL<br>MIC = 25.0–350.0 µL/mL | [69]       |
|                   | Ripe umbels, Portugal                                             |                                                                              | <i>Bacillus subtilis</i> , <i>Listeria monocytogenes</i> , and <i>Staphylococcus aureus</i> .<br><i>Escherichia coli</i> and <i>Salmonella typhimurium</i> .                                                                                                                                                  | MIC = 0.32–0.64 µL/mL<br>MIC > 10 µL/mL                                                                                                                                      | [66]       |
|                   | Leaves, flower, petals, and fruits; Uzbekistan                    |                                                                              | <i>Bacillus subtilis</i> and <i>Staphylococcus aureus</i> .                                                                                                                                                                                                                                                   | MIC = 6–7 µL/mL                                                                                                                                                              | [71]       |
|                   | The aerial parts, France                                          | Essential oil (vapor distillation)                                           | Inhibitory effects against <i>C. jejuni</i> , <i>C. coli</i> , and <i>C. lari</i> .<br>Inhibitory effects against <i>C. jejuni</i> F38O11, LV7, and LV9 clinical isolates vs. human isolate LV11.<br>Inhibitory effects against <i>C. jejuni</i> LM7A.<br>Inhibitory effects against <i>C. jejuni</i> Lme27A. | MIC = 250 µg/mL<br>MIC = 250 µg/mL vs. MIC = 500 µg/mL<br>MIC = 250 µg/mL<br>MIC = 125 µg/mL                                                                                 | [5]        |
|                   | Umbels, Tunisia                                                   | Essential oil (hydrodistillation) and supercritical CO <sub>2</sub> extracts | Inhibitory effects against <i>Escherichia coli</i> ATCC 35218 and <i>Staphylococcus aureus</i> ATCC 43300.                                                                                                                                                                                                    | MIC > 2.5% (v/v)                                                                                                                                                             | [9]        |
|                   | Umbels, Lebanon                                                   | <i>Daucus carota</i> (DC) aqueous and methanolic extracts                    | Inhibitory effects against <i>Staphylococcus aureus</i> meti S and meti R:<br>Aqueous extract;<br>Methanolic extract.                                                                                                                                                                                         | MIC = 20 mg/mL<br>MIC = 10 mg/mL                                                                                                                                             | [97]       |
|                   | Flowers and roots, Tunisia                                        | Essential oil (hydrodistillation)                                            | Inhibitory effects against Gram-positive and Gram-negative bacteria.<br>Flower oil: more effective against <i>E. coli</i> (ESLβ).<br>Root oil: more effective against <i>S. aureus</i> , <i>S. pneumoniae</i> , <i>Shigella</i> spp., and <i>E. faecalis</i> .                                                | MIC = 1.25–5 mg/mL<br>MIC = 1.25 mg/mL<br>MIC = 1.25 mg/mL                                                                                                                   | [76]       |
| <i>hispanicus</i> | Roots and aerial parts, Algeria                                   |                                                                              | Aerial part oil:<br>Inhibitory effects against <i>B. subtilis</i> ;<br>Inhibitory effects against <i>S. aureus</i> .<br>Root oil:<br>Inhibitory effects against <i>B. subtilis</i> ;<br>Inhibitory effects against <i>S. aureus</i> .                                                                         | MIC = 1.2 mg/mL<br>MIC = 4.8 mg/mL<br>MIC = 1.5 mg/mL<br>MIC = 4.2 mg/mL                                                                                                     | [77]       |
| <i>maximus</i>    | Green seeds, Italy                                                | Essential oil (steam distillation)                                           | Inhibitory effects against Gram-positive strains ( <i>Staphylococcus</i> and six out of twenty-five <i>L. monocytogenes</i> strains) and Gram-negative strains ( <i>Acinetobacter</i> and <i>Stenotrophomonas maltophilia</i> ICE272).                                                                        | MIC = 1.25–2.5 µL/mL                                                                                                                                                         | [75]       |

### 6.3. Antifungal Activity

The antifungal properties of *D. carota* subspecies were studied against different fungal strains. *D. carota* ssp. *carota*, *gummifer*, *halophilus*, *hispanicus*, and *maximus* were found

to exhibit significant antifungal activities. Oil extract derived from different plant organs of *D. carota* ssp. *carota* demonstrated both fungistatic and fungicidal activities, with *Fulvia fulvum* being the most susceptible and *Trichoderma viride* and *Aspergillus ochraceus* being the most resistant. Among the plant organs, the oil from unripe fruits exhibited the strongest antifungal activity, followed by ripe fruit oil, root oil, stem oil, leaf oil, and flower oil [69]. In 2017, Asilbekova et al. [71] showed that fruit essential oil of *D. carota* ssp. *carota* from Uzbekistan showed antifungal activity against *C. albicans*. However, the essential oils of *D. carota* ssp. *carota* umbels from Poland displayed weak inhibitory activity against *Candida albicans* and *Penicillium expansum* [8]. Furthermore, the antifungal activity of *D. carota* ssp. *carota* blooming and flowering umbels' essential oils from Sardinia and Portugal as well as ripe umbels from Portugal were investigated against yeast, dermatophytes, and filamentous fungi [6,66]. The oils demonstrated greater antifungal potential against *Cryptococcus neoformans* and dermatophyte strains compared to *Candida* and *Aspergillus* strains. The mechanism of action involves an interference with preformed biofilms, reducing the amount of attached biomass and inhibiting germ-tube formation. According to the authors, the antifungal effect was attributed to the presence of  $\alpha$ -pinene, geranyl acetate, and  $\alpha$ -limonene [66]. Previous reports indicated that  $\alpha$ -pinene displayed antifungal activity against several *Candida albicans* strains [54] as well as other fungal species such as *C. neoformans* and *R. oryzae* [59,100]. The antifungal activity was mainly attributed to an interference with biofilm formation.  $\alpha$ -limonene has also been shown to possess significant antifungal properties against different fungal strains, such as *Sclerotinia sclerotiorum*, *Zygosaccharomyces rouxii*, *Trichophyton rubrum*, *Candida*, and yeast [101–106]. Moreover, geranyl acetate was found to have a broad antifungal spectrum against different dermatophyte strains, *Cryptococcus neoformans*, and *Candida guilliermondii* [107].

The antifungal properties of other *D. carota* subspecies were also investigated. In 2015, Valente et al. [24] revealed that an essential oil from aerial parts of *D. carota* ssp. *gummifer* was found to be more active against *C. guilliermondii*, dermatophyte strains, and *C. neoformans* compared to other *Candida* strains. The authors attributed the antifungal properties to the high contents of geranyl acetate and  $\alpha$ -pinene. The essential oil from aerial parts of Portuguese *D. carota* ssp. *maximus*, also tested for antifungal activity, demonstrated a broad antifungal spectrum against many fungal strains, especially *Aspergillus*, *Candida*, dermatophytes and *Cryptococcus neoformans* [74]. The oil also exhibited a higher antifungal activity against *Aspergillus* and *Candida* strains compared to other Portuguese subspecies and various *D. carota* ssp. *taxa* from different countries [8,69]. Similarly, the authors named  $\alpha$ -pinene and geranyl acetate as the main contributors to these antifungal effects. In another study by Tavares et al. [70], essential oils from umbels of *D. carota* ssp. *halophilus* were shown to possess significant antifungal activity against dermatophyte strains, linked to the presence of elemicin. In addition, an essential oil extracted from roots of *D. carota* ssp. *hispanicus* was found to display a stronger antifungal effect against *A. flavus* than an oil extracted from the aerial parts, and these effects were ascribed to the presence of the phenolic derivatives myristicin and apiole [77].

These findings highlight the moderate broad antifungal properties of *D. carota* ssp. *carota*, *hispanicus*, *maritimus*, and *maximus*. As such, the oil of these various *D. carota* spp. may be recommended as an antifungal remedy that could be used in traditional medicine, as no previous claims of such activity have been reported. Further studies are required to confirm these findings using in vitro and in vivo models. It is also important to identify the active ingredients responsible for this antifungal property and to elucidate the mechanism of action involved. Table 7 summarizes the antifungal effects of *D. carota* ssp. *carota*, *gummifer*, *halophilus*, *hispanicus*, and *maximus*.



**Table 7.** In vitro antifungal effects of *D. carota* subspecies.

| DC Subspecies     | Plant Organ, Location                                             | Treatment                                                                    | Detail                                                                                                   | Concentration/MICs                                                            | References            |      |
|-------------------|-------------------------------------------------------------------|------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|-----------------------|------|
| <i>carota</i>     | Ripe and unripe fruits, flowers, roots, leaves, and stems; Serbia | Essential oil (hydrodistillation)                                            | Inhibitory effects against <i>Fulvia fulvum</i> ;                                                        | MIC = 2.0–100.0 μL/mL                                                         | [69]                  |      |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>Trichoderma viride</i> ;                                                   | MIC = 25.0–100.0 μL/mL                                                        |                       |      |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>Aspergillus ochraceus</i> .                                                | MIC = 10.0–150.0 μL/mL                                                        |                       |      |
|                   | Herbs and umbels, Poland                                          |                                                                              | Inhibitory effects against <i>Candida albicans</i> ;                                                     | MIC = 5 μL/mL                                                                 | [8]                   |      |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>Penicillium expansum</i> .                                                 | MIC = 8 μL/mL                                                                 |                       |      |
|                   | Umbels, Tunisia                                                   |                                                                              | Inhibitory effects against clinical strains of <i>Candida albicans</i> and <i>C. tropicalis</i> 1011 RM. | MIC > 2.5% ( <i>v/v</i> )                                                     | [9]                   |      |
|                   | Ripe umbels, Portugal                                             |                                                                              | Inhibitory effects against <i>Cryptococcus neoformans</i> ;                                              | MIC = 0.16 μL/mL                                                              | [66]                  |      |
|                   |                                                                   | Inhibitory effects against dermatophytes;                                    | MIC = 0.32–0.64 μL/mL                                                                                    |                                                                               |                       |      |
|                   |                                                                   | Inhibitory effects against <i>C. guilliermondii</i> .                        | MIC = 0.32 μL/mL                                                                                         |                                                                               |                       |      |
|                   |                                                                   | Fruits, Uzbekistan                                                           |                                                                                                          | Inhibitory effects against <i>Candida albicans</i> .                          | MIC = 12 μL/mL        | [71] |
|                   | Blooming and flowering umbels, Sardinia Islands and Portugal      | Essential oil (hydrodistillation) and supercritical CO <sub>2</sub> extracts | Portugal:                                                                                                |                                                                               | [6]                   |      |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>Cryptococcus neoformans</i> ;                                              | MIC = 0.32–0.64 μL/mL                                                         |                       |      |
|                   |                                                                   |                                                                              | Inhibitory effects against dermatophytes.                                                                | MIC = 1.25–2.5 μL/mL                                                          |                       |      |
|                   |                                                                   |                                                                              | Italy:                                                                                                   |                                                                               |                       |      |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>Cryptococcus neoformans</i> ;                                              | MIC = 0.32 μL/mL                                                              |                       |      |
|                   |                                                                   |                                                                              | Inhibitory effects against dermatophytes.                                                                | MIC = 0.16–0.32 μL/mL                                                         |                       |      |
| <i>gummifer</i>   | Aerial parts, Portugal                                            | Essential oil (hydrodistillation)                                            | Inhibitory effects against dermatophyte strains and <i>C. neoformans</i> ;                               | MIC = 0.32–0.64 μL/mL                                                         | [24]                  |      |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>C. guilliermondii</i> .                                                    | MIC = 1.25 μL/mL                                                              |                       |      |
| <i>halophilus</i> | Umbels, Portugal                                                  |                                                                              | Inhibitory effects against dermatophytes.                                                                | MIC = 0.16–0.64 μL/mL                                                         | [73]                  |      |
| <i>hispanicus</i> | Root and aerial parts, Algeria                                    |                                                                              | Inhibitory effects against <i>Candida albicans</i>                                                       | MIC = 0.078 ± 0.02 mg/mL                                                      | [77]                  |      |
|                   |                                                                   |                                                                              | Aerial parts:                                                                                            | MIC = 0.125 ± 0.04 mg/mL                                                      |                       |      |
|                   |                                                                   |                                                                              | Roots:                                                                                                   |                                                                               |                       |      |
|                   |                                                                   |                                                                              |                                                                                                          | Inhibitory effects against <i>Aspergillus flavus</i> .                        |                       |      |
|                   |                                                                   |                                                                              |                                                                                                          | Root vs. aerial parts oil inhibition:                                         | 100% vs. 42.22%       |      |
| <i>maximus</i>    | Aerial parts, Portugal                                            |                                                                              |                                                                                                          | Inhibitory effects against dermatophytes and <i>Cryptococcus neoformans</i> ; | MIC = 0.16–0.32 μL/mL | [74] |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>Aspergillus</i> ;                                                          | MIC = 0.32–0.64 μL/mL                                                         |                       |      |
|                   |                                                                   |                                                                              | Inhibitory effects against <i>Candida</i> .                                                              | MIC = 0.64–1.25 μL/mL                                                         |                       |      |

#### 6.4. Antioxidant Activity

Free radicals are known to contribute to several ailments, including cardiovascular, cancer, and degenerative diseases. Numerous antioxidant studies have been conducted on *D. carota* ssp. *carota*, with only one study on *D. carota* ssp. *gummifer*. Akgul et al. [108] reported that oil extracted from *D. carota* L. ssp. *carota* flowers from Turkey possesses significant DPPH radical scavenging properties, and this effect was stronger than that of individual compounds extracted from the oil. Similarly, Shebaby et al. [37] indicated antioxidant properties for the Lebanese *D. carota* ssp. *carota* umbel oil extract (DCOE) using the FRAP and DPPH assays. The results exhibited significant radical scavenging activity of DCOE in comparison to Trolox. The authors attributed the scavenging role of DCOE to the high levels of terpenes, phenols, and polyphenolic compounds. Additionally, an essential oil from the Algerian wild carrot flower demonstrated the strongest antioxidant activity compared to the aerial part essential oils before and after flowering. The

antioxidant capacity of the oils was due to the presence of monoterpenes such as  $\alpha$ -pinene,  $\beta$ -bisabolene, sabinene, and limonene [67]. Another study by Ksouri et al. [68] reported that the methanolic and essential oil of Algerian *D. carota* ssp. *carota* exhibit antioxidant activities in different in vitro assays, including DPPH and TBARS assays. However, the essential oil of *D. carota* ssp. *carota* ripe umbels from Portugal exhibited weak antioxidant effects using ABTS and peroxy-induced oxidation inhibition assays [66]. On the other hand, a *D. carota* ssp. *gummifer* aerial parts' essential oil displayed a substantial nitric oxide (NO) scavenging effect in all tested concentrations [24].

In vitro and in vivo antioxidant properties of DCOE fractions were also investigated [109]. The diethyl ether and chloroform/methanol fractions demonstrated the highest antioxidant activity in DPPH and FRAP assays. These effects could be attributed, at least in part, to the presence of phenolic compounds such as luteolin, kaempferol, apigenin, caffeic acid, and quercetin, all of which have been reported to display antioxidant properties [110–114].

The antioxidant properties of different fractions of DCOE were examined both in vitro and in living organisms in vivo [109]. The fractions made using diethyl ether and chloroform/methanol were found to have the strongest antioxidant activity when tested using the DPPH and FRAP assays. This activity is likely due to the presence of various phenolic compounds, including luteolin, kaempferol, apigenin, caffeic acid, and quercetin, which are known to have antioxidant properties [110–114]. On the other hand, the pentane fraction that is abundant in sesquiterpenes displayed moderate antioxidant activity when evaluated by DPPH assay. This can be linked to the presence of  $\alpha$ -humelene and  $\beta$ -caryophyllene, which are known to have significant antioxidant effects [82,115–117]. Additionally, the sesquiterpene  $\alpha$ -longipinene, found in pentane, diethyl ether, and chloroform/methanol fractions, was reported to exhibit antioxidant effects [118,119]. Furthermore, the pentane and pentane/diethyl ether fractions were highly effective at binding and neutralizing  $\text{Fe}^{2+}$  ions (strong chelating activity). Metal ions such as  $\text{Fe}^{2+}$  play a crucial role in the formation of hydroxyl radicals through the Fenton reaction, accelerating lipid peroxidation [120].  $\beta$ -Caryophyllene, a prevalent sesquiterpene found in the pentane fraction, has been reported as a potent inhibitor of lipid peroxidation due to its ability to react with peroxy radicals [121]. The antioxidant properties of DCOE fractions were also evaluated in vivo by measuring the activity of enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST) in the livers of mice intoxicated with  $\text{CCl}_4$ . The pentane fraction (200 mg/kg) and the chloroform/methanol fraction (50, 100, and 200 mg/kg) were found to increase CAT and SOD activity; however, the pentane/diethyl ether and diethyl ether fractions were ineffective in restoring the enzymes' activity to normal levels [109]. All these studies describe the multimodal mechanism of action of *D. carota* in combating free radicals, highlighting its role as a prominent antioxidant agent.

### 6.5. Anti-Inflammatory Activity

Wild carrot has traditionally been used in folk medicine to treat various inflammatory conditions like cystitis and prostatitis. In light of this, researchers have conducted numerous studies to examine the anti-inflammatory properties of various *D. carota* ssp. including spp. *carota*, *gummifer*, and *maximus*. Both the aqueous (100, 200, and 400 mg/kg BW) and methanolic (70, 140, and 280 mg/kg BW) extracts of the Lebanese *D. carota* ssp. *carota* umbels displayed a significant reduction in acute and chronic inflammation in rats. The anti-inflammatory effects observed were similar to those of the non-steroidal anti-inflammatory drug (NSAID) diclofenac and were associated with the presence of terpenes and flavonoids in the plant extracts [97]. Additionally, research has found that an essential oil extracted from the aerial parts of *D. carota* ssp. *gummifer* possesses potent anti-inflammatory properties. It was able to inhibit the production of nitric oxide (NO) in macrophages and microglia cultures that were stimulated with lipopolysaccharide (LPS) [24]. Similar studies have also been conducted to investigate the effects of an essential oil from the aerial parts of *D. carota* ssp. *maximus* on the NO production in LPS-stimulated macrophages and microglia cells. At a non-toxic concentration (0.32  $\mu\text{L/mL}$ ), the essential

oil was able to decrease NO production by 20.7% and 35.8% in macrophages and microglia cells, respectively. The anti-inflammatory properties of the oil were partly attributed to the presence of high levels of geranyl acetate and  $\alpha$ -pinene [74]. Multiple studies have also confirmed the anti-inflammatory effects of these two terpenes. In particular, (+)- $\alpha$ -pinene was found to be effective in inhibiting the IL-1 $\beta$ -stimulated inflammatory and catabolic pathways, thus exhibiting potential anti-osteoarthritic activity [122]. It also reduced IL-6 and TNF- $\alpha$  formation in macrophages of rats and decreased nitrite production [123]. On the other hand, geranyl acetate showed significant anti-inflammatory activity in vitro, without affecting macrophages' and keratinocytes' viability [107]. Despite the relatively high doses used in the above studies, the findings align with the traditional use of wild carrot as an anti-inflammatory remedy, especially considering the use of a crude extract. Additional in vivo studies can further support its traditional use.

#### 6.6. Miscellaneous

In a study by Wehbe et al. [97], the researchers examined the ability of aqueous and methanolic extracts of Lebanese *D. carota* ssp. *carota* umbels to protect against ethanol-induced gastric damage in rats. Both extracts demonstrated a protective effect, with the methanolic extract showing a higher curative ratio compared to the aqueous extract and the group treated with cimetidine. According to these authors, this gastro-protective effect is likely due to the presence of flavonoids and tannins in the plant. Additionally, the aqueous extract was found to decrease levels of HDL, without having any significant impact on total cholesterol, triglycerides, and LDL concentrations. Furthermore, no significant effects were observed on the glucose, insulin, and amylase activity in the blood after the use of the aqueous extract. In another study by Muturi et al. [72], it was revealed that the essential oil of *Daucus carota* ssp. *carota* displayed significant toxicity against mosquito larvae, suggesting the potential use of wild carrot essential oils as a bio-pesticide. The contraceptive effects of the wild carrot have been investigated as well. Several in vivo studies have shown that extracts from wild carrot seeds can impede pregnancy through diverse mechanisms, including acting as anti-zygotic and/or blastocystotoxic agents [124] and inhibiting implantation through anti-progestogenic and antioestrogenic effects [125–127].

### 7. Safety/Toxicological Evaluation

In addition to the assessment of their efficacy, subspecies *carota*, *gummifer*, *halophilus*, and *maximus* were evaluated for their safety. The aqueous extract of Lebanese *D. carota* ssp. *carota* umbels showed no significant variations in liver enzyme concentrations (SGOT, SGPT, ALP, and LDH) in rats, suggesting that the extract can maintain the structural integrity of the hepatocellular membrane [97]. Tawil et al. [25] revealed an extremely low susceptibility of normal human peripheral blood mononuclear cells (PBMCs) to DCOE methanol:acetone (1:1) treatment. Additionally, according to Dixon's up and down model, the LD<sub>50</sub> of the major compound  $\beta$ -2-himachalen-6-ol (HC), obtained from Lebanese *D. carota* ssp. *carota* umbels, was 1000 folds higher than that of cisplatin, highlighting the low toxicity profile of DCOE in adult Balb/c mice [64]. In another study, chronic treatments with HC displayed mild hepatotoxicity with no adverse effects on the kidneys of the treated mice. The HC-treated groups showed the highest survival rate among all groups [95]. Furthermore, a study using a 1,2-dimethylhydrazine (DMH) colon carcinogenesis model in six black mice found that HC did not cause significant liver toxicity at doses of 10 and 25 mg/kg, while moderate hepatotoxicity was observed at a concentration of 50 mg/kg [94]. Daaboul et al. [93,96] also reported that no body weight changes were observed after HC treatment in contrast to cisplatin, which exhibited significant weight reductions in the animals. No variations in liver enzyme profiles were reported after HC treatment, indicating no hepatotoxicity. On the other hand, the essential oil of *D. carota* ssp. *carota* derived from ripe umbels from Portugal was tested on keratinocytes, alveolar epithelial cells, macrophages, and hepatocytes [66]. The oil was considered safe at concentrations be-

low 0.64  $\mu\text{L/mL}$ , with macrophages being the least vulnerable ( $92.83\% \pm 1.04$  cell viability) and hepatocytes the most susceptible ( $60.73\% \pm 6.51$  cell viability).

A previous report indicated that an essential oil derived from the aerial parts of *D. carota* ssp. *gummifer* does not affect the cell viability of macrophages at concentrations below 1.25  $\mu\text{L/mL}$  [24]. Comparable results were observed for liver cells (hepatocytes) and skin cells (keratinocytes). However, it was somewhat more toxic to microglia cells, leading to decreased cell viability when used at concentrations above 0.64  $\mu\text{L/mL}$  [24].

When testing *D. carota* ssp. *halophilus* umbel essential oils at concentrations possessing significant antifungal activity, no cytotoxicity was detected in mouse skin dendritic cells [70]. Lastly, the safety of the essential oil from aerial parts of Portuguese *D. carota* ssp. *maximus* was assessed on human keratinocytes and hepatocytes as well as mouse macrophages and microglial cells [74]. The results showed that cell viability at concentrations lower than 1.25  $\mu\text{L/mL}$  was not affected for all cell lines except microglial cells, where cytotoxicity was observed at a concentration as low as 0.64  $\mu\text{L/mL}$ .

## 8. Discussion and Conclusions

Traditional medicine has always been the backbone of drug discovery, and with the accelerating need for new and improved medication options, an in-depth study of the pharmacological potential of natural remedies is essential. As previously reported, the wild carrot has a long history of traditional use for treating a variety of conditions, including pain, stomach ulcers, diabetes, cancer, inflammation, and infections [5,11–13].

To better characterize the therapeutic potential of wild *D. carota*, it is essential to investigate the interplay between its traditional uses and pharmacological activities in relation to its chemical composition. As previously cited in this review, *D. carota* ssp. *carota* has a longstanding traditional use in Lebanon to fight oncological ailments, a usage substantiated by numerous studies underscoring its significant anticancer properties, attributed mainly to the presence of the distinctive compound  $\beta$ -2-himachalen-6-ol [25,37,64,65,89–96]. The wild carrot has also been traditionally employed by the Lebanese and Persian communities in the treatment of gastric ulcers [40,43], as supported by a study indicating that both aqueous and methanolic extracts possess protective effects against ethanol-induced gastric damage in rats. The authors attribute this gastro-protective activity to the presence of flavonoids and tannins within the plant [97]. Furthermore, in another investigation, the plant extract exhibited hepatoprotective effects in the livers of  $\text{CCl}_4$ -intoxicated mice by increasing or restoring the normal levels of the antioxidant liver enzymes [109]. These findings offer additional support for the use of wild carrots in traditional folk medicine for the protection against hepatic diseases [40].

Regarding the antimicrobial activities, various subspecies, including *carota*, *hispanicus*, *maritimus*, and *maximus*, have exhibited mild to moderate antibacterial properties against various bacterial species, aligning with their historical use in folk medicine as antiseptics for treating bacterial infections, such as prostatitis and cystitis [5,11–13]. Notably,  $\alpha$ -pinene and geranyl acetate emerged as key constituents contributing to this antibacterial efficacy, which is in line with other studies that emphasize the antibacterial potential of  $\alpha$ -pinene [51–60]. While folk medicine does not explicitly mention the use of the wild carrot for fungal infections, multiple studies underscore its significant antifungal activities against diverse fungal strains, particularly within *D. carota* ssp. *carota*, *gummifer*, *halophilus*, *hispanicus*, and *maximus* [6,8,24,66,69,70,74,77]. The phytochemicals responsible for this antifungal activity, namely,  $\alpha$ -pinene, geranyl acetate, and  $\alpha$ -limonene, align with findings from related studies [54,100–107]. In addressing inflammatory conditions, *D. carota* ssp. *carota*, *gummifer* and *maximus* have demonstrated noteworthy anti-inflammatory properties [24,74,97] that were mainly attributed to the presence of  $\alpha$ -pinene and geranyl acetate [107,122,123]. This aligns with the historical utilization of wild carrots in folk medicine for the treatment different inflammatory disorders like pain, prostatitis, and cystitis [5,11–13]. The observed anti-inflammatory effect of the wild carrot may also be attributed to the existence of antioxidant compounds in the plant extract [24,37,66,67,108,109]. Notably, various terpenes including  $\alpha$ -pinene,  $\beta$ -Caryophyllene,  $\beta$ -bisabolene, sabinene, limonene, and  $\alpha$ -longipinene along with

polyphenols like luteolin, kaempferol, apigenin, caffeic acid, and quercetin are recognized for their radical scavenging activities [110–114,118,119,121]. An elevated production of reactive oxygen species (ROS) is linked to oxidative stress and the oxidation of proteins [128]. This, in turn, triggers inflammatory mediators and numerous inflammatory signals in response to protein oxidations [129]. Moreover, an excess of ROS can induce tissue injury, activating the inflammatory process [130,131]. Hence, the well-established antioxidant properties of the diverse terpenes and polyphenols present in the wild carrot play a crucial role in their anti-inflammatory effects by disrupting the cycle of ROS-induced inflammation. These findings reinforce the convergence of scientific research with traditional practices, elucidating the specific entities responsible for desired effects. Other pharmacological properties of the wild carrot also confirm its practice in traditional medicine. Based on several in vivo studies, various mechanisms may contribute to the anti-fertility effect of the wild carrot, including an impact on the estrous cycle and anti-progestogenic activity [125–127]. These results further emphasize the ethnopharmacological practices of the wild carrot, both as a contraceptive agent and in its capacity as an emmenagogue [27,33,34].

Critically reviewing the literature available on the wild carrot reveals few limitations in current studies. To begin with, searching for *D. carota* in the literature revealed that authors do not always specify the subspecies that they refer to in their studies, which is an essential piece of information due to intraspecific polymorphism of these species. Usually, when referring to “*D. carota*”, authors target one single *D. carota* ssp. *sativus*, the most known, cultivated, and edible subtype. Therefore, it is important to point out the confusion that overshadows the nomenclature of the carrot. A correct naming and description of the subspecies must be employed by scholars for more accurate and credible research referencing. Another common mistake resides in not specifying the plant organ studied, namely, whether the authors used the whole plant or specific organs (leaves, roots, stems, flowers, etc.), and this creates confusion among readers. Moreover,  $\alpha$ -pinene, geranyl acetate,  $\beta$ -2-himachalen-6-ol, and  $\alpha$ -limonene are the main chemical constituents that are highlighted to exhibit significant pharmacological activities in their respective studies. However, other important components, such as myristicin, apiole, and dillapiole, have not been studied in this context, although they are present abundantly in different plant organs.

In conclusion, this review underscores the pharmacological merits of the wild carrot supported by its historical use in folk medicine and achieved through the synergistic interaction of specific essential chemical constituents. Nevertheless, certain applications outlined in traditional medicine remain unexplored. Therefore, there is a necessity to investigate the potential role of the wild carrot in conditions such as diuresis, toxicosis, kidney stones, bladder and gastric diseases, parasitic infections, and its potential as an antidote for snake bites. Furthermore, the current review specifies the chemical composition of various subspecies and identifies key phytochemicals responsible for their medicinal properties. *D. carota* ssp. *carota* from Lebanon emerges as the most studied, displaying unique anti-cancer activity. While almost all subspecies exhibit significant antibacterial, antifungal, antioxidant, and anti-inflammatory activities, further in vivo and pre-clinical studies are imperative to confirm these findings, ensuring the safety and efficacy of *D. carota* in diverse therapeutic applications. Moreover, the isolation and characterization of biologically active compounds and mechanistic elucidation should be prioritized for a more comprehensive understanding of the plant’s pharmacological properties.

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