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Bioactivity of Medicinal Plants and Extracts

Edited by
Francisco Les, Víctor López and Guillermo Cásedas

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About the Editors

Francisco Les

Graduated in Veterinary Medicine from University of Zaragoza in 2012, Graduated in Pharmacy from San Jorge University (USJ) in 2015 and Doctor in Health Sciences also from USJ in 2017.

Professor of the degrees in Pharmacy and Nursing in subjects of Pharmacology, Plant Biology, Pharmacognosy and Phytotherapy.

His line of research is focused on investigating plant extracts and active ingredients of plant origin of pharmaceutical, cosmetic and agri-food interest, studying the bioactivity and toxicity of new compounds in in vitro or in vivo models.

He has published more than 40 articles on plant or food extracts and active ingredients with pharmacological and nutritional interest. He has participated in national and international conferences on pharmacy, herbal medicine, physiology, diabetes and obesity.

He defended a doctoral thesis with international mention entitled "Study of the bioactive properties of pomegranate juice and other compounds of polyphenolic origin", obtaining the qualification of cum laude and the Extraordinary Award of the Doctoral Program in Health Sciences. In addition, this has been awarded the first prize for doctoral theses in the areas of phytotherapy, medicinal plants, nutritherapy and natural ingredients of the "Arkopharma Institute Doctoral Thesis Awards 2021".

During his academic training, he has received six scholarships to support research and the subsequent completion of his doctorate, and has carried out international research stays at the "Dipartimento di Neuroscienze"in Florence (Università di Firenze, Italy) in 2017, and at the "Institute of Cardiovascular and Metabolic Diseases"(Université Toulouse III, France) in 2015.

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Pharmacist and nutritionist with a PhD in Pharmaceutical Sciences currently working as Associate Professor and Vicedean at Universidad San Jorge in Spain. Involved in teaching and researching in the field of pharmacology, natural products and functional foods.

His line of research focuses on the pharmacology of natural products with particular emphasis on those bioactive compounds, natural antioxidants or plant extracts of pharmaceutical, cosmetic or agri-food interest. Head of Phyto-Pharm, a multidisciplinary and international research group with experience in the development of competitive projects for public calls but also through contracts with companies in the pharmaceutical sector.

Author of more than 75 articles in indexed international journals with numerous communications in specialized congresses, as well as several book chapters.

Other merits: collaborating researcher at the Aragon Agrifood Institute (IA2), member of the Board of Directors of the Spanish Society of Phytotherapy (SEFIT), member of the Spanish Society of Pharmacology, and member of the Kingdom of Aragon Academy of Pharmacy.

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His research line is focused on the study of active ingredients of plant origin and plant extracts of pharmaceutical and food interest. Mainly, in vitro pharmacological assays of enzyme inhibition (AChE, MAO-A, Tyrosinase) and antioxidant activity (DPPH radical, superoxide radical, ORAC), quantification of endogenous antioxidant systems (catalase, SOD, GPx, GR), oxidative stress (ROS) and protein expression in cellular models (glial and neuronal).

He is the author of the chapter "Engineering and Biomedical Effects of Commercial Juices of Berries, Cherries, and Pomegranates With High Polyphenol Content"of the book Non-alcoholic beverages Volume 6: The Science of Beverages (Elsevier).

He has published more than 20 articles on isolated bioactive compounds and polyphenols presented in plants in indexed scientific journals. He has participated and presented more than 20 communications in national and international conferences on phytotherapy, physiology, nutrition, pharmacology and pharmacy, oxidative stress and diabetes.

He is a peer reviewer for various scientific journals related to antioxidants and natural products.

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Editorial

Bioactivity of Medicinal Plants and Extracts

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Nature is an inexhaustible source of bioactive compounds and products with interesting medicinal properties and technological applications. Although natural products can be found in plants, animals, microorganisms and minerals, the vast majority of them come from plants [1].

Since the beginning of the ages, plants have produced a great variety of molecules through different biosynthetic routes. Some of them are considered essential for the normal performance and development of the plant, such as carbohydrates, lipids and proteins; this aggregate is called primary metabolites [2].

The biochemical pathways also lead to the production of relatively small molecules known as secondary metabolites. These secondary metabolites do not seem essential for plant development. However, science has demonstrated that secondary metabolites have important functions in plants, for instance, defence against ultraviolet radiation exposure; struggling against infections caused by viruses, fungi, bacteria and phytopathogens; or keeping herbivores away. These secondary metabolites are the most interesting in therapeutics and belong to three large groups known as polyphenols, terpenes and alkaloids [3].

Natural products have constituted the origin of pharmacology and therapeutics. Early on, they were used as medicinal plants or preparations, and later as isolated molecules or phytochemically characterized extracts. Plants are still a source in nature for obtaining and isolating molecules with pharmacological applications (drug discovery), but can also be used as herbal medicinal products in traditional or complementary medicine. In addition, the WHO has launched a Traditional Medicine Strategy (2014–2023), including herbal medicines as medicinal therapies, with the aim of ensuring the quality, safety, proper use and effectiveness of traditional medicines, among other objectives [4].

More recently, natural products have continued to enter clinical trials or to provide leads for compounds that have entered clinical trials, particularly as anticancer and antimicrobial agents. Further, research in natural products has shown many advantages [5,6]:

— Nature presents a chemical diversity that is practically immeasurable by human mind.
— Natural compounds, by virtue of being biosynthesized by living organisms, are expected to be "drug-like" because they have already had to interact with enzymes, receptors and biological signalling pathways.
— Bioactive compounds that come from species used in traditional medicine have a greater probability of success because they are already being used for therapeutic purposes.

Throughout history, human have used plants for therapeutic purposes; the development of synthetic and organic chemistry allowed herbal medicines to be replaced by isolated molecules provided by the pharmaceutical industry, even though approximately 50% of them are not completely synthetic and have a natural origin [7–9]. It is also important to note that research on natural products has increased exponentially in recent years, but the percentage of new approved drugs that have a natural origin has decreased. This fact has been caused by different factors, such as aspects of intellectual property, respect for biodiversity, accessibility to living organisms or the amount of active substance available in nature [10,11].

In addition to their nutritional, industrial, ecological and environmental value, plants have played (and still continue to play) a crucial role in medicine and pharmacy [12].

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Article

Essential Oil of *Foeniculum vulgare* Mill. as a Green Fungicide and Defense-Inducing Agent against Fusarium Root Rot Disease in *Vicia faba* L.

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Simple Summary: Plant extracts, including essential oils, are a viable alternative method for controlling plant diseases. This work deals with the exploitation of fennel seed essential oil (FSEO) to inhibit *Fusarium solani* and control *Fusarium* root rot disease in *Vicai faba*. In vitro FSEO inhibited mycelium growth by up to 80% at 400 µL/mL of FSEO. In vivo, the protective effects against *Fusarium* root rot disease were recorded when FSEO was applied to *Vicia faba* seeds. The FSEO reduced the disease severity from 98% in plants grown in infested soil with *Fusarium solani* to 60.1% in plants that previously had their seeds treated with FSEO. GC-MS spectrometry analyses showed that the major chemical components in the essential oil were D-limonene, menthol, estragole and 2-decenal. Applications of the essential oil resulted in increased total phenolic and flavonoid contents in leaves compared with untreated inoculated (control) plants. The defense-related genes, such as defensin and chitinase, were differentially expressed. This study revealed that the essential oil of fennel seed was effective as a control agent against *Fusarium* root rot in broad beans.

Abstract: *Fusarium solani*, the causative agent of root rot disease is one of the major constraints of faba bean (*Vicia faba* L.) yield worldwide. Essential oils have become excellent plant growth stimulators besides their antifungal properties. *Foeniculum vulgare* Mill. (fennel) is a familiar medicinal plant that has inhibitory effects against phytopathogenic fungi. Herein, different concentrations of fennel seed essential oil (FSEO) (12.5, 25, 50, 100, 200 and 400 µL/mL) were examined against *F. solani* KHA10 (accession number MW444555) isolated from rotted roots of faba bean in vitro and in vivo. The chemical composition of FSEO, through gas chromatography/mass spectroscopy, revealed 10 major compounds. In vitro, FSEO inhibited *F. solani* with a minimum inhibitory concentration (MIC) of 25 µL/mL. In vivo, FSEO suppressed *Fusarium* root rot disease in *Vicia faba* L. by decreasing the disease severity (61.2%) and disease incidence (50%), and acted as protective agent (32.5%) of *Vicia faba* L. Improvements in morphological and biochemical parameters were recorded in FSEO-treated faba seeds. Moreover, the expression level of the defense-related genes defensin and chitinase was noticeably enhanced in treated plants. This study suggested using FSEO as a promising antifungal agent against *F. solani* not only to control root rot disease but also to enhance plant growth and activate plant defense.

Keywords: *Foeniculum vulgare*; chitinase; defensin; qRT-PCR; *Vicia faba*; plant disease; root rot; essential oil; plant promotion

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1. Introduction

Vicia faba L., commonly known as faba bean or broad bean, is an economical legume grain that widely contributes to human consumption, animal fodder and silage making [1]. Faba beans have high nutritional value due to their high protein content, minerals, vitamins and considerable amounts of bioactive compounds [2]. This crop is usually planted at the end of summer. *Vicia faba* is susceptible to several diseases that reduce their yield, especially in moist conditions. Faba beans are attacked by a common disease known as root rot, which is caused by many fungal species including *Fusarium solani*, *Rhizoctonia solani* and *Sclorotium rolfsi* [2–4]. *Fusarium solani* is one of the most pathogenic fungi that deteriorate the quality and quantity of many crops' production [5]. *F. solani* is a common plant pathogen that invades a wide range of hosts including 111 plant species belonging to 87 genera [6]. Globally, *Vicia faba* L. is one of the most common plants suffering from root rot disease. *Fusarium* root rot takes place at the beginning of the growing season, resulting in the death of seedlings [4]. Otsyula et al. [7] reported that, the yield of common beans decreased by up to 84% due to root rot induced by *F. solani*. Management of *Fusarium* root rot is a complex task due to the soil-borne pathogens being located near the rhizosphere and their long-term survival by producing resistant spores [8]. Although there are fungicides that can control fungal diseases, they have negative impacts on human health, the ecosystem and evolving fungicide-resistant strains [9]. Therefore, the use of natural alternatives is the only reliable source for controlling plant diseases [10]. Essential oils (EOs) are among the most important alternatives that play a vital role in plant protection and food preservation, with a wide variety of applications. Several studies have confirmed the use of EOs to manage plant pathogenic fungi and improve the safety and quality of crops [11,12]. The chemical composition of EOs contains many bioactive molecules that have antifungal, antibacterial and antioxidant activity [13]. EOs have many antagonistic effects against bacteria and fungi, as they drive the plasma membrane to lose its ability to act as a barrier, followed by the release of intracellular components and suppression of cellular respiration with homeostasis failure [14]. Moreover, EOs can inhibit the formation of fungal cell walls and electron transport in the mitochondria [15]. Remarkably, one of the important actions of EOs on the plasma membrane is to suppress the secretion of toxins [16]. The usage of EOs effectively enhances the safety and quality of cereals and food products [11]. Regardless of antimicrobial activity, applying essential oils as biocides has many benefits beside antimicrobial activity, including pre-harvest restrictions, being non-toxic for human health, being reliable to use in any type of lands and their convenience for all types of cultivation, such as organic systems [11]. Herbs and aromatic plants are usually used for medicinal purposes and for inhibiting microbes, since they contain essential oils [17]. The fennel herb (*Foeniculum vulgare* Mill.), a member of the *Apiaceae* family, is an important medicinal plant found almost all over the world [18]. It is used as a carminative, antiseptic, diuretic, digestive and expectorant, aiding anticancer, anti-inflammatory, antimicrobial, and antioxidant activities [19]. The leaves and fruits of fennel are used in cosmetics and flavoring substances, while seed extracts exhibit antifungal effects against *Aspergillus* sp., *Candida* sp., *Sclerotinia sclerotiorum* and many other phytopathogens and dermatophytes [18,19]. The main components of fennel oil are trans-anethole (53.51%), carvacrol (11.93%), fenchone (8.32%) and thymol (8.11%) [20]. The essential oil of *F. vulgare* is enriched with phytochemicals such as polyphenols that give it antimicrobial and antioxidant properties [21]. The use of EOs can move from pathogen suppression to plant protection, presumably by different strategies; one such mechanism is defensin, a term used in the description of antimicrobial and antifungal proteins (AFPs), isolated from mammals, insects and plants, and serving as effectors molecules of innate immunity, providing an efficient initial defense against infectious pathogens [22]. Another such strategy is the synthesis of pathogenesis-related (PR) proteins such as chitinases, which hydrolyze chitin, a linear polymer of β-1,4-linked *N*-acetylglucosamine residues that is one of the primary cell wall components of many pathogenic fungi [23,24]. The level of expression of PR genes such as chitinase and defensin increases the defensive response

of plants against a wide range of pathogens [25]. Although FSEO has been used to inhibit some fungal species, several studies are still required to discover its efficiency against fungal plant pathogens and plant diseases, and its effect on plant quality and resistance. In addition, there is an increasing demand for this green, natural and safe product for future approaches to crop protection and organic farming. In this context, this study planned to investigate the antifungal potential of FSEO as a fungicide to overcome the economically damaging *Fusarium* root rot disease of *Vicia faba* L. in vitro and in vivo. Furthermore, the effect of FSEO was assessed on plant growth, antioxidant enzymes and the expression levels of defensin genes in *Vicia faba* L.

2. Materials and Methods

2.1. Plant Material and Extraction of Fennel Seed Essential Oil

Foeniculum vulgare (fennel) seeds were obtained from a local market (El-Hawag Company, Cairo, Egypt), and identified and authenticated by the Department of Botany, Faculty of Science, Mansoura University, Egypt. Fifty grams of fennel seeds was air dried and ground into a fine powder and then placed in a 2000 mL flask with 500 mL of distilled water and extracted by the hydro-distillation process using a Clevenger-type apparatus for 5 h to extract the essential oils. The oils were collected in a 250 mL conical flask, dried over anhydrous sodium sulphate and kept at 4 °C until use [26].

2.2. Gas Chromatography/Mass Spectral Analysis

The chemical composition of the essential fennel oil was determined by gas chromatography-mass spectrometry system (GC-MS-QP 2010, Shimadzu, Japan), equipped with a flame-ionization detector (FID) with a Rtx-5MS column (30 m × 0.25 mm, 0.25 µm thickness). The essential oil (10 µL) was dissolved in acetone (100 µL) and 1 µL of the solution was injected into the GC/MS system with the following properties: helium was the carrier gas, used at a flow rate of 1 mL/min; split mode (1:25), with 1 µL (1/10 in acetone, *v*/*v*) as the injected volume and 300 °C as the injection temperature. The mass spectra of the obtained compounds were matched with those in the NIST11 library (Gaithersburg, MD, USA) [27].

2.3. Isolation of Fusarium solani, Pathogenicity Test and Cultivation

Fusarium solani was isolated in the laboratory from infected roots of faba bean plants (*Vicia faba* L.) displaying external signs of rot root disease. The plants were collected from agricultural areas in the production region of Behera, Egypt, in the winter of 2019. *F. solani* isolation was achieved by cutting the infected root into pieces (2 to 3 mm). The fragments were surface-sterilized with a 10% sodium hypochlorite solution for 2 min, then rinsed with sterile distilled water ternary. Pieces were cultured aseptically onto a *Fusarium* selective medium—Nash-Snyder agar (1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$-$7H_2O$, 15 g/L peptone, 20 g/L agar, 1 g/L pentachloronitrobenzene, 0.3 g/L streptomycin sulfate, 0.12 g/L neomycin sulfate) [28]—and incubated at 25 ± 2 °C for 5–7 days. The fungal mycelium was sub-cultured on Czapek-Dox agar medium (CZA) (30 g/L sucrose, 3 g/L $NaNO_3$, 0.5 g/L KCl, 100 mg/L $FeSO_4$-$7H_2O$, 0.5 g/L $MgSO_4$-$7H_2O$, 1 g/L K_2HPO_4). Morphological features as well as microscopic characteristics were investigated [29]. Moreover, molecular identification was also applied; the universal primers ITS1/ITS2 for the ribosomal internal transcribed spacer (ITS) were used. The sequence was compared with the suggested species using the BLAST sequence analysis tool and was registered into GenBank under the accession number MW444555. Koch's postulate was implemented to confirm that the symptoms of root rot belonged to *F. solani* KHA10 [30]. Eventually, cultures attained from single spores were maintained on CZA and kept at 4 °C for further use. The pure culture has been placed in the culture collection of the Botany and Microbiology Department, AUC (No. BMS0023).

2.4. In Vitro Evaluation of Antifungal Activity and Growth Inhibition

2.4.1. Agar Well Diffusion Method

The antifungal activity of FSEO was tested by the well diffusion method with minor modifications. *F. solani* was inoculated on a Czapek-Dox (CZ) broth medium and then incubated at 25 ± 2 °C for 5–7 days [13]. Fungal inoculum of *F. solani* was spread on the surface of CZA plates. Next, 5 wells 8 mm in diameter were made using a sterile cork-borer on each agar plate (90 mm). The wells were filled with 100 µL of different concentrations of FSEO. Basically, 3 mL of Tween 80 was mixed with 97 mL of sterile distilled water. FSEO at 25, 50, 100, 200 and 400 µL/mL was prepared by adding 25, 50, 100, 200 and 400 mL of FSEO each to 1 L of sterile distilled water and Tween 80 (3%), respectively [31]. The culture plates were incubated at 25 °C for 7 days, and the zones of inhibition were observed and measured. All experiments were performed in triplicate.

2.4.2. Radial Growth Method

Radial growth of *F. solani* was evaluated at different concentrations of FSEO (25, 50, 100, 200 and 400 µL/mL) according to method used by Hashem et al. [1], with minor changes. The fennel essential oil was mixed well with the molten CZA medium at the desired final concentrations. Different concentrations of essential oil were prepared by dissolving the required amounts in sterile CZA amended with Tween 80 (0.1%, v/v) to obtain the desired concentrations (25, 50, 100, 200, 400 µL/mL). The medium was then poured into Petri dishes and kept until solidifying. The center of each plate was inoculated with a mycelium plug (6 mm diam.) from a 7-day-old culture, and the plates were then incubated at 25 ± 2 °C. Mycelium growth was assessed daily by measuring the diameters of the colony in each plate. Inhibition percentage of pathogen growth was calculated using the following equation:

$$\text{Inhibition of pathogen growth } (\%) = \frac{\text{Growth in the control} - \text{Growth in the treatment}}{\text{Growth in the control}} \times 100$$

2.5. Pot Experiment

2.5.1. Preparation of Fungal Inoculum

The inoculum of *F. solani* KHA10 was prepared based on Büttner et al. [32] with slight modification as follows: a 500 mL sterilized Erlenmeyer flask containing 250 mL of the sterilized CZ medium was inoculated with 3 discs (5 mm in diameter) from the edge of 5-day-old *Fusarium* culture, and then incubated in the dark for 7 days at 25 ± 2 °C under shaking (125 rpm). Conidiospores were counted using a hemocytometer, and the inoculum suspension was adjusted to a final concentration of 10^6 spores/mL. The inoculum was kept chilled at 4 °C until use.

2.5.2. Fennel Seeds, Growth Conditions and Treatments

Seeds of *Vicia faba* L. (Nubaria1) were obtained from the Agriculture Research Center (ARC), Ministry of Agriculture, Egypt. The *Vicia faba* seeds were washed with distilled water then sterilized using 2% sodium hypochlorite for 2 min. *Vicia faba* seeds were grown in plastic pots (15 cm in diameter × 15 cm in depth), previously sterilized using a 5% formaldehyde solution and filled with 1 kg of sterile sandy clay soil (4:1). Two weeks before planting, the soil was infested with *F. solani* KHA10. Soil infestation was carried out by adding 90 mL of a 10^6 spore/mL suspension of *F. solani* KHA10/pot. The infested soil was kept moist for 7 days to stimulate fungal growth and ensure homogeneous distribution of the fungus. The control treatment was prepared by adding the same amount of the sterilized Czapek-Dox broth (CZ) to the sterilized soil of each pot. The pots were grown in greenhouse conditions at 25 ± 5 °C, with a 14 ± 2 h light regimen and humidity at 65 ± 10%, and irrigated as necessary. Treatments used in this study were as follows: (1) healthy control (C)—the sterilized *Vicia faba* seeds submerged in distilled water (D.W.) for 6 h and sowing in sterilized soil; (2) treated with FSEO (T)—the sterilized *Vicia faba* seeds

soaked in FSEO 400 µL/mL at the minimal fungal concentration (MFC) for 6 h and sown in sterilized soil; (3) control infected with *F. solani* (P)—sterilized *Vicia faba* seeds soaked in D.W. for 6 h and sown in soil previously inoculated with *F. solani*; (4) seeds treated with FSEO (T+P), sterilized *Vicia faba* seeds soaked in FSEO 400 µL/mL for 6 h and sown in soil previously inoculated with *F. solani*. The data were collected at 3 intervals (3 weeks, 6 weeks and 10 weeks). The results collected after 3 weeks of growth were much like the control because there was not much time for plant to be affected by both the pathogen and the treatments. After 10 weeks, it was very hard to collect data because the plant was old and affected too much by the pathogen to create a huge variation in the results (Supplementary Figure S1, Tables S1–S3). Five seeds/pot of *Vicia faba* for each treatment were applied. All experiments were arranged in a completely randomized split-plot design with 3 replicates per treatment. Six weeks after planting, all pots were evaluated for the incidence of *Fusarium* root rot. Percentages of seed rot, pre- and post-emergence damping off, and plant survival were also recorded [33].

2.6. Disease Assessments

Disease severity (DS) and incidence (DI) of *Fusarium* root rot were assessed in *Vicia faba* L. 6 weeks after planting. Disease severity was evaluated using the 0–5 scale described by [34].

$$\text{Disease severity (\%)} = \sum ab/AK \times 100$$

where a = number of diseased plants with the same infection degree, b = infection degree, A = total number of the evaluated plants and K = the greatest infection degree.

Disease incidence (DI) of *Fusarium* root rot was assessed pre-emergence and at post-emergence damping off after the treatments. Disease incidence was calculated for each treatment according to the following equation:

$$\text{Disease incidence (\%)} = a/A \times 100$$

where, a = number of diseased plants and A = total number of evaluated plants.

2.7. Analysis of Plant Growth Parameters

Samples were assessed after 6 weeks of sowing. The morphological traits of treated and untreated faba bean plants were measured. Three plants with *Fusarium* root rot from each experiment were harvested and transferred to the laboratory, carefully uprooted and washed using tap water for measuring plant height, and shoot and root fresh and dry weight. For dry weight, samples were oven-dried at 40 °C for 48 h.

2.8. Biochemical Analyses

For each treatment, 3 plants were collected 30 days after treatment and analyzed for total phenol content (TPC), total flavonoid content (TFC), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and antioxidant enzymes.

2.8.1. Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The scavenging activity of DPPH radicals was evaluated by adding a 1 mM solution of DPPH in ethanol to 1.5 mL (1 mg/L mL) of the EO extract solution. The freshly prepared DPPH solution was taken in test tubes and extracts were added, followed by serial dilutions (100–1000 µg) in every test tube such that the final volume was 2 mL, and the absorbance was evaluated at 517 nm against the corresponding blank solution, which was prepared by taking 3 mL ethanol, and the control O.D. was prepared by taking 3 mL of DPPH. The assay was repeated 3 times. DPPH percentage inhibition was estimated based on the control reading [1].

$$\text{DPPH scavenged (\%)} = (\text{A cont.} - \text{A test})/\text{A cont.} \times 100$$

where A cont. is the absorbance of the control reaction and A test is the absorbance in the presence of the sample of the extracts.

2.8.2. Total Phenolic Content

Total phenols were measured in the uppermost leaves using the ethanol extraction method (80%, v/v); the supernatant was added to Folin and Ciocalteau's reagent as described [35].

2.8.3. Total Flavonoid Content

Total flavonoid content ($mg \cdot g^{-1}$ fresh weight) was measured using aluminum chloride catechin equivalent (CAE) as the standard accordingly [35].

2.8.4. Phenylalanine Lyase Assay

PAL activity was determined following the method described by Whetten and Sederoff [36]. The mixture of the assay, including 500 µL 50 mM Tris HCI and 100 µL plant extract, (pH 8.8), and 600 µL 1 mM L-phenylalanine, was incubated at room temperature for 1 h, and 2 N HCI was used to stop the reaction. Toluene (1.5 mL) was used to extract the assay mix by vertexing for 30 s. After centrifugation at 300 g, toluene was recovered for 5 min using a CRU-5000 centrifuge ITC. The toluene phase (containing trans-cinnamic acid) absorbance was measured at 290 nm. The enzyme activity was expressed as nmol trans-cinnamic acid released min^{-1} g^{-1} fresh weight.

2.8.5. Polyphenol Oxidase (PPO)

Extraction of PPO was performed as reported by [37]. Powdered samples (0.5 g) were homogenized with a buffer containing 20 mL of a 100 mM sodium phosphate buffer (pH 7.0) and 0.5 g polyvinyl pyrrolidone (PVP) (mol. wt 40,000) for the assay of the activity of PPO. The activity was measured in powder extracted with a 50 mM sodium phosphate buffer (pH 8.8) containing 5 mM β-mercaptoethanol. The extracts were filtered through 2 layers of Mira cloth, and the filtrates were centrifuged at 27,000× g at 4 °C for 30 min.

2.8.6. Antioxidant Enzyme Quantification

Samples (500 mg) of leaves were homogenized in a 50 mM KH_2PO_4 buffer (pH 7.8) with 0.1 mmol L^{-1} EDTA, 0.1% (v/v) Triton X-100 and 2% PVP, and centrifuged at 4 °C for 10 min at 22,000× g. The supernatant obtained was reserved for the assays of the different antioxidants.

The activities of total superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) were recorded as follows: SOD activity was evaluated based on Kono (1978) [38] by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reduction of NBT was followed by an absorbance increase at 540 nm in a reaction mixture containing 1.3 mL Na-carbonate buffer (50 mM, pH 10.0), 500 µL NBT (96 µM) and 100 µL Triton X-100 (0.6%). The reaction was initiated by the addition of 100 µL hydroxylamine-HCl (20 mM, pH 6.0); 2 min later, 70 µL of the enzyme sample was added. The enzyme activity was calculated as the SOD concentration inhibiting the reduction of NBT by 50%. CAT activity was measured based on the method described by Aebi (1974) [39]. The rate of decomposition of H_2O_2 was superseded by a decrease in absorbance at 240 nm in a reaction mixture containing 1.5 mL K-phosphate buffer (100 mM, pH 7.0), 1.2 mL H_2O_2 (150 mM) and 300 µL of the enzyme extract. Enzyme activity was estimated by the extinction coefficient of 6.93×10^{-3} mM^{-1} cm^{-1}. Moreover, APX activity was measured based on the method of Nakano and Asada (1981) [40] achieved by a decrease in absorbance at 290 nm in a reaction mixture containing 1.5 mL K-phosphate buffer (100 mM, pH 7.0), 300 µL ascorbate (5 mM), 600 µL H_2O_2 (0.5 mM) and 600 µL of the enzyme extract. Enzyme activity was determined using the extinction coefficient of 2.8 mM^{-1} cm^{-1}, and was calculated as the amount of enzyme required to oxidize 1 µmol of ascorbate min^{-1} g^{-1} tissue.

2.8.7. Expression of Defense-Related Genes

Total RNA was extracted from 0.5 g fresh faba leaves at 1, 2 and 3 weeks after sowing from all treatments and the control using an RNA extraction kit (QIAGEN, Redwood, CA, USA). The obtained RNA was incubated with DNase for 1 h at 37 °C and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). An RT-PCR kit (Omniscript RT; QIAGEN) was used for the synthesis of cDNA. Thermo QuantStudio 12K Flex Real-Time PCR System qRT-PCR was carried out in triplicate with 3 biological repeats using TOP real TM qPCR 2X Pre MIX SYBR Green (Enzynomics, Daejeon, Korea) according to the manufacturer's instructions using the given primers of the defense-related genes defensin and chitinase (Table 1) using β-actine as the reference gene. The PCR cycle was: 95 °C for 5 min (hot-start activation) followed by 40 cycles of 95 °C for 10 s (denaturation), 58 °C for 20 s (annealing) and 72 °C for 20 s (extension). The melting curve was generated after 40 cycles to test the specificity of each primer pair across the temperature range of 60–95 °C at a heating rate of 0.05 °C/s. Gene expression analyses were performed according to Rawat et al. [41].

Table 1. Primers used for qRT-PCR defense gene analysis.

Primer	Primer Sequence	Annealing Temp.
Chitinase-F	5′-GCGGATCCCAACGCACTGCAACCGATTAT-3′	60 °C
Chitinase-R	5′-GCCCATGGAAGGAATCAGTTATGCGCAAAT-3′	
Defensin-F	5′-CCAAATGCCTCGTCATCT-3′	
Defensin-R	5′-ATTAGAGTCAAGCTCAAAAGG-3′	
β-Actin	5′-GTGGGCCGCTCTAGGCACCAA-3′	
β-Actin	5′-CTCTTTGATGTCACGCACGATTTC-3′	

2.9. Statistical Analysis

Data Procession System (DPS) was used for analysis of variance (ANOVA). Two-way ANOVA was used to test the effect of E and P and their interactions on plant health, followed by the least significant difference (LSD). Correlation, PCA analysis and presentation were performed using R version 3.4.2.

3. Results

3.1. Chemical Composition of Fennel Oil

Since the researchers did not know the mechanism behind the antifungal activities of fennel seeds, this study attempted another experiment to check the chemical composition of fennel seeds and whether it could lead us to a significant result. Therefore, the chemical composition of fennel oil was inspected through gas chromatography/mass spectrometry (GC/MS) analysis, which revealed the presence of 10 major compounds in different percentages. The most abundant compound was cis-vaccenic acid (31.23%), followed by 9,12 octadecadienoic acid (29%), pentadecanoic acid (7.51%), estragole (4.39%), octadecadienoic acid (3.92%); 9-octadecadienoic acid (3.75%), D-limonene (2.93%), menthol (1.89%), 2,4-decadienal (1.68%) and 2-decenal (1.58%) (Figure 1 and Table 2).

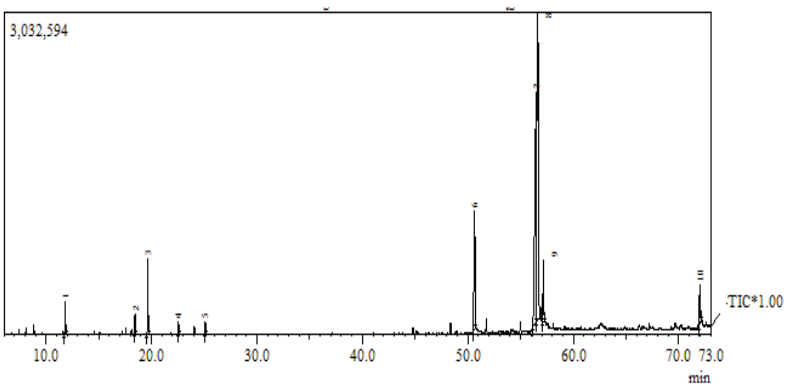

Figure 1. Chromatogram: GC-MS chromatogram of *Foeniculum vulgare* essential oils.

Table 2. Chemical composition of *Foeniculum vulgare* oil by GC-MS analysis.

Quantitative ID	Component Identified	Retention Time (min)	Area (%)
1	D-limonene	11.81	2.93
2	Menthol	18.44	1.89
3	Estragole	19.66	4.39
4	2-Decenal	22.51	1.58
5	2,4-Decadienal	25.1	1.68
6	Pentadecanoic acid	50.64	7.51
7	9,12-Octadecadienoic acid	56.45	29.49
8	Cis-vaccenic acid	56.63	31.23
9	Octadecadienoic acid	57.15	3.92
10	9-Octadecadienoic acid	72	3.75

3.2. In Vitro Control of F. Solani by Fennel Essential Oils

3.2.1. Antifungal Activity and Minimum Inhibitory Concentration of FSEO

The antifungal activity of fennel seeds was investigated at different concentrations (25, 50, 100, 200 and 400 µL/mL) to inhibit *Fusarium solani* KHA10, the causative agent of *Fusarium* root rot disease in *Vicia faba* by the agar well diffusion method (Figure 2). The results revealed that all concentrations of FSEO showed antifungal activity against *F. solani* KHA10. However, 400 µL/mL presented the most antifungal activity, with a 38 mm inhibition zone, while 25 µL/mL exhibited the lowest antifungal activity, inhibiting the growth of *F. solani* with a 1 mm inhibition zone. According to the previous results, 25 µL/mL of FSEO was the MIC for controlling *F. solani*.

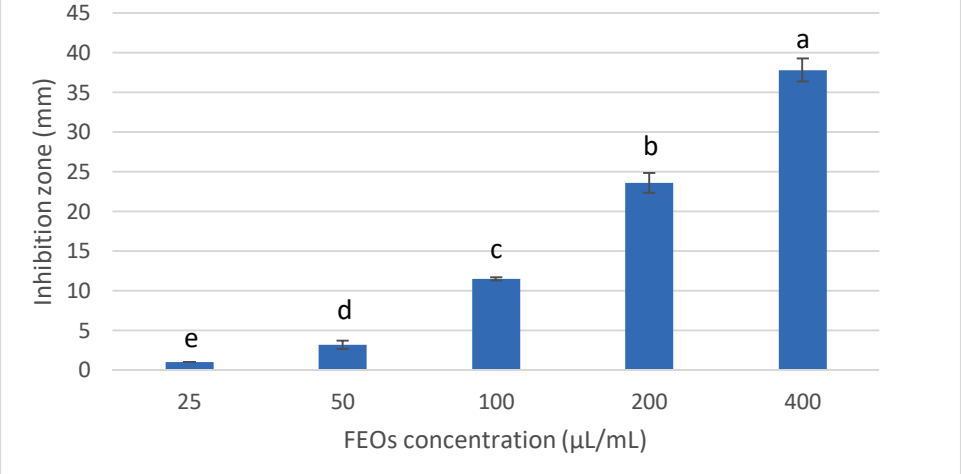

Figure 2. Antifungal activity of FSEO at different concentrations against *F. solani*. Data are expressed as means ± standard deviations in triplicate. Different alphabetic superscripts in the same column are significantly different ($p < 0.05$) based on Tukey's multiple comparison test.

3.2.2. Effect of FSEO on the Radial Growth of *F. solani* and Minimum Fungicidal Concentrations

In vitro, the antifungal activity of different concentrations of fennel seed essential oil extract was tested against the mycelial growth of *F. solani* KHA10 with different incubation periods from 1 to 7 days (Figure 3A–C). The radial growth was examined to measure the inhibition percentage of each FSEO concentration. The results demonstrated that the inhibition percentage increased with an increasing concentration of FSEO, while the radial growth ceased, as shown in Figure 3B. *Fusarium solani* could not grow at 400 µL/mL on the CZA surface, with an inhibition percentage of 100%, so this concentration had the minimum fungicidal activity (Figure 3). Moreover, FSEO at 25 µL/mL allowed the growth of *F. solani* with only 6% inhibition percentage.

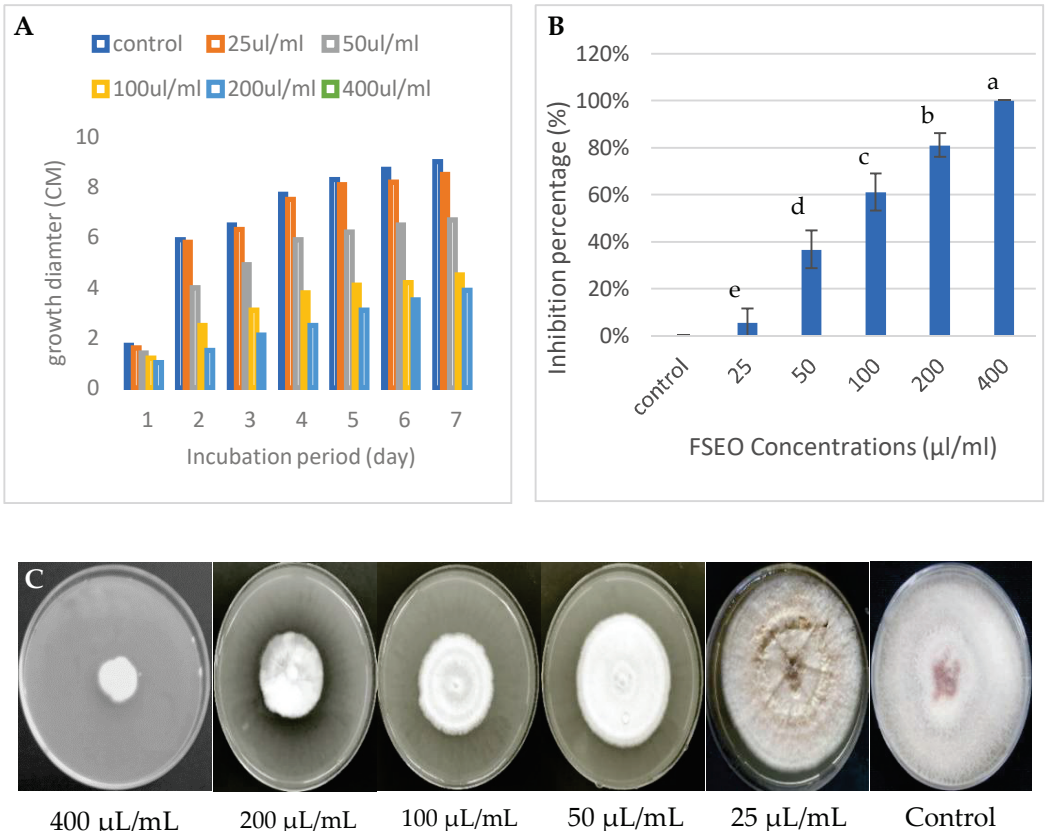

Figure 3. Effect of FSEO on *F. solani* (**A**–**C**): (**A**) radial growth at different incubation periods from 1 to 7 days; (**B**) inhibition percentages of *F. solani* at different concentrations of FSEO; (**C**) radial growth on Czapek-Dox agar medium (CZA) plates at 7 days. CZA not supplemented with FSEO was used as a control. Data are expressed as means ± standard deviations of triplicate assays. Different alphabetic superscripts in the same column are significantly different ($p < 0.05$) based on Tukey's multiple comparison test.

3.3. In Vivo Control of F. solani KHA10 by Fennel Essential Oil

Efficacy of FSEO on *Fusarium* Root Rot Disease of *Vicia faba* L. under Pot Conditions

After applying the treatments to *Vicia faba*, morphological and disease progression were observed at the pre-emergence, post-emergence and 6 week stages (Table 2). Pre- and post-emergence, the damping off was decreased by 25% and 19%, respectively. The seeds exposed to the pathogen and FSEO (P+E) were much healthier compared were seeds treated with the pathogen (P) only. In addition, the P-only treated seeds clearly developed the disease.

The growth performance of FSEO-treated plants were much improved over the control (C) and P plants after 6 weeks of plantation. Broad bean plants treated with P+E were taller and healthier than P plants. The results showed that the disease resistance was higher in the 400 µL/mL FSEO treated plants.

After 400 µL/mL FSEO treatment of *Vicia faba* L. seeds, disease incidence (DI) and disease severity (DS) decreased significantly as compared with the pathogen-inoculated seeds only at 6 weeks after planting. The percentage of DI in faba seeds soaked with 400 µL/mL FSEO decreased to 33.5% as compared with pathogen-only infected plants. Plant survival and protection were clearly improved when FSEO was applied to infected seeds by approximately 44% and 50%, respectively (Table 3).

Table 3. Mean *Fusarium* root rot incidence and severity at pre- and post-emergence damping off after different treatments were applied to *Vicia faba* L.

Treatments	Pre-Emergence Damping off %	Post-Emergence Damping off %	Survival Plant %	Disease Severity (DS) (%)	Disease Incidence (DI) %	Protection %
Healthy control (C)	0	0	100	0	0	-
Treated with FSEO (T)	0	0	100	0	0	-
Infected control (P)	46.5	33.8	19.71	53.1	67.4	0
Treated +infected (T+P)	21.8	14.5	63.7	20.6	33.5	32.5

3.4. Physiological Characterization of FSEO Treated Faba Bean Plants

The physiological characterization data of greenhouse application treatments showed a significant increase in the growth parameters of *Vicia faba* plants, viz. plant height (P.h), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW) and root dry weight (RWD), by soaking seeds in 400 µL/mL FSEO, compared with *F. solani* KHA10 inoculation. Maximum P.h was recorded in the case of FSEO: 49.92 cm at 6 weeks after sowing. The control was second in rank, where a P.h of 43.20 cm was recorded, while T+P recorded a P.h. of 39.50 cm. As expected, the lowest P.h was observed in the case of the pathogen treatment: 36.70 cm at 6 weeks after sowing. Overall, SFW, RFW, SDW and RDW were significantly higher in the T+P treatment than in the pathogen-only treatment. Apart from that, we also observed a significant in of SFW, RFW, SDW, and RDW in the oil-treated plants as compared with the controls (Table 4).

Table 4. Effect of FSEO and *F. solani* KHA10 on morphological parameters of *Vicia faba* L. in pot conditions at 6 weeks of treatment.

Treatments	Plant Height (cm)	Shoot F. wt. (g)	Root F. wt. (g)	Shoot D. wt. (g)	Root D. wt. (g)
Healthy control (C)	43.2 ± 0.18^{d}	4.37 ± 0.02^{b}	2.16 ± 0.03^{a}	0.56 ± 0.03^{a}	0.34 ± 0.07^{a}
Treated with FSEO (T)	49.92 ± 1.2^{a}	4.75 ± 0.03^{a}	2.43 ± 0.03^{a}	0.66 ± 0.02^{b}	0.38 ± 0.06^{b}
Infected control (P)	36.7 ± 1.0^{b}	2.69 ± 0.02^{b}	1.59 ± 0.02^{b}	0.38 ± 0.01^{c}	0.24 ± 0.02^{c}
Treated + infected (T+P)	39.4 ± 0.52^{c}	3.27 ± 0.02^{b}	1.74 ± 0.03^{a}	0.41 ± 0.01^{d}	0.28 ± 0.01^{d}

Values are the means of 15 replicates ± standard errors. Values in each column followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

3.5. Influence of FSEO on Different Biochemical Parameters

The second part of this study was the biochemical analysis of fennel seed extract and its influence on 2,2-diphenyl-1-picrylhydrazyl (DPPH), total phenolic contents (TPC), total flavonoid contents (TFC), phenylalanine ammonia lyase (PAL) and polyphenyl oxidase (PPO) (Figure 4). Significantly high DPPH was detected for *Vicia faba* L. treated with FSEO, which measured 57.05 µg g^{-1} dry wt. 6 weeks after sowing, while the control recorded 49.97 µg·g^{-1} dry wt. (Figure 4A). Pathogen-infected *Vicia faba* L. was strongly affected, where DPPH was 12.77 µg g^{-1} dry wt., illustrating the strong influence of the pathogen on faba bean plants. A significant decrease in DPPH values was reported in the case of FSEO+P, namely 18.16 µg g^{-1} dry wt., explaining the role of FSEO in plants defense against fungal diseases (Figure 4A).

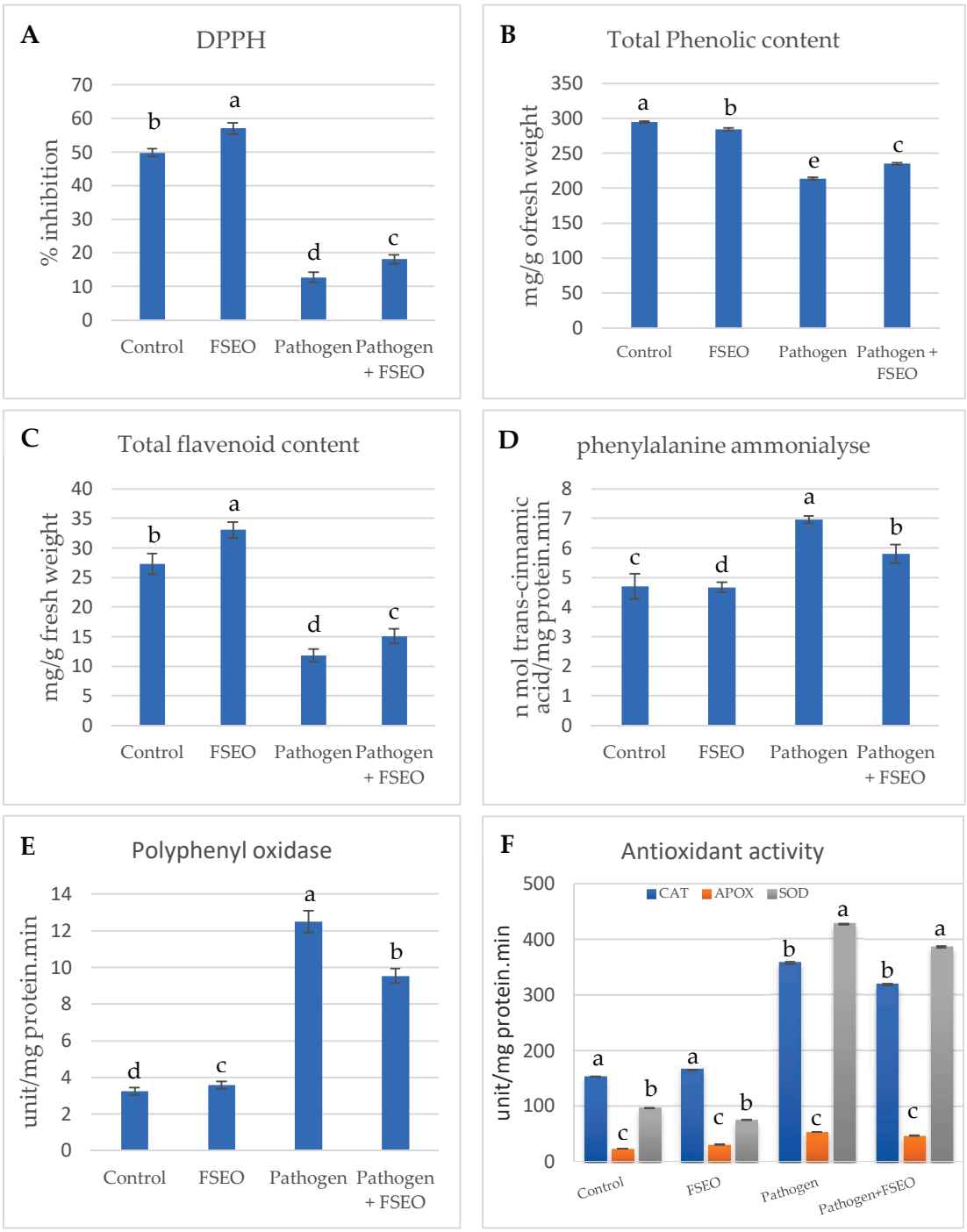

Figure 4. Biochemical components and antioxidant enzymes of plants, after 6 weeks of treatment. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (**A**), total phenol content (TPC) (**B**), total flavonoid content (TFC) (**C**), phenylalanine ammonia lyase (PAL) (**D**), polyphenol oxidase (PPO) (**E**) and antioxidant enzymes (**F**). Error bars indicate ± standard error of the mean of three replicates. Different alphabetic superscripts in the same column are significantly different ($p < 0.05$) based on Tukey's multiple comparison test.

Total phenolic content is another biochemical contributor in plants that has redox properties, acting as an antioxidant (Figure 4B). The highest TPC was detected in control plants 6 weeks after sowing, recording 294.68 mg catechol 100 g^{-1} dry wt., while in the case of the FSEO treatment, the recorded TPC was 284.34 mg catechol 100 g^{-1} dry weight. In the case of FSEO+P, the recorded TPC was 235.13 mg catechol 100 g^{-1} dry wt. Total

phenolic content dramatically decreased to 213.83 mg catechol 100 g^{-1} dry wt. when plants were treated with *F. solani* (pathogen) (Figure 4B).

The level of total flavonoid content increased in *Vicia faba* L. treated with FSEO to 33.06 mg rutin 100 g^{-1} dry wt. at 6 weeks after sowing, while the control was 27.29 mg rutin 100 g^{-1} dry wt. The treatment of faba beans with FSEO +P recorded TFC at 15.09 mg rutin 100 g^{-1} dry wt., while the level of TFC was significantly decreased at 11.84 mg rutin 100 g^{-1} dry wt. in the case of the pathogen treatment (Figure 4C).

Additionally, the highest assay of PAL was detected when *Vicia faba* L. was treated with pathogen: 7.00 nM cinnamic g^{-1} fresh wt. 6 weeks after sowing, while PAL was at 4.7 nM cinnamic g^{-1} fresh wt. in the case of the control. PAL was at 5.81 nM cinnamic g^{-1} fresh wt. when faba bean was treated with FSEO +P, whereas PAL was at 4.67 nM cinnamic g^{-1} fresh wt. in the case of FSEO (Figure 4D).

Regarding polyphenol oxidase (PPO), the data showed a significantly increase PPO 12.50 µg g^{-1} dry wt. when *Vicia faba* L. was treated with *F. solani*. Moreover, PPO was at 9.55 µg g^{-1} dry wt. in case of FSEO +P, while FSEO only recorded 3.57 µg g^{-1} dry wt. The smallest amount of PPO, 3.24 µg g^{-1} dry wt., was recorded with the control (Figure 4E).

In addition, DI showed a strong negative ($p \leq 0.01$) correlation with biochemicals such as DPPH, TPC and TFC, illustrating that an increase in disease incidence or severity will lead to a decrease in these biochemical or plant physiological characteristics and vice versa. However, there was a strong positive ($p \leq 0.01$) correlation between DI with antioxidants, showing that a parallel increase or decrease in one will affect the other component positively (Figure 5).

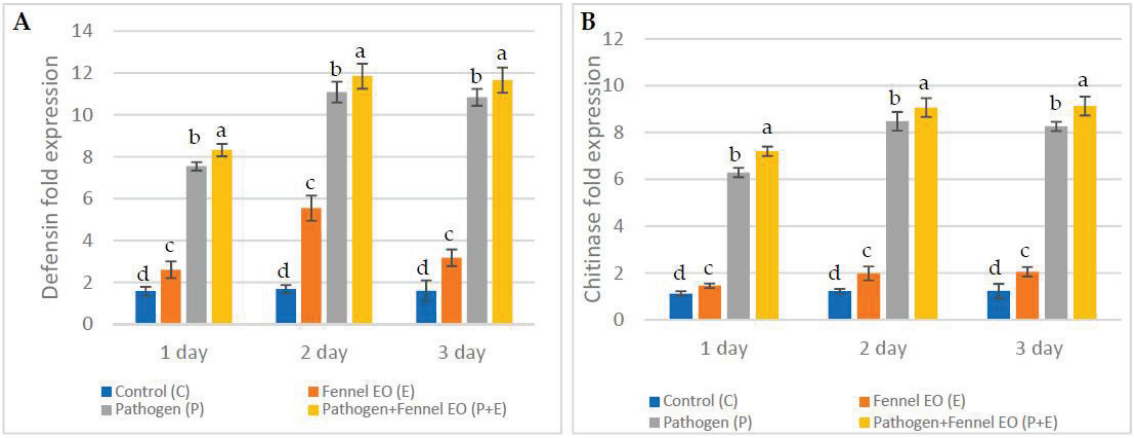

Figure 5. Fold expression in the accumulation of the defense-related genes (**A**) defensin and (**B**) chitinase in *Vicia faba* L. samples, at different treatments relative to the control, and at different periods. Values are the means (±SD) of three repeated experiments. Different alphabetic superscripts in the same column are significantly different ($p < 0.05$) based on Tukey's multiple comparison test.

Concurrently, we also performed PCA analysis to identify the relationship of variables at 6 weeks in plants grown under different treatments. Correlations between variables were found via biplot analyses, where an acute angle means a positive correlation, an obtuse angle means a negative correlation and a right angle means no correlation between the measured parameters. The first principal component has the largest variance due to the orthogonal transformation. According to the PCA calculated for all the data, the first factor (PC1) explained 71.5% of the total variance of the variables, and the second factor (PC2) about 24.2%. In total, both PCs explained 95.7% of the total variance of all the analyzed variables (Figure 6).

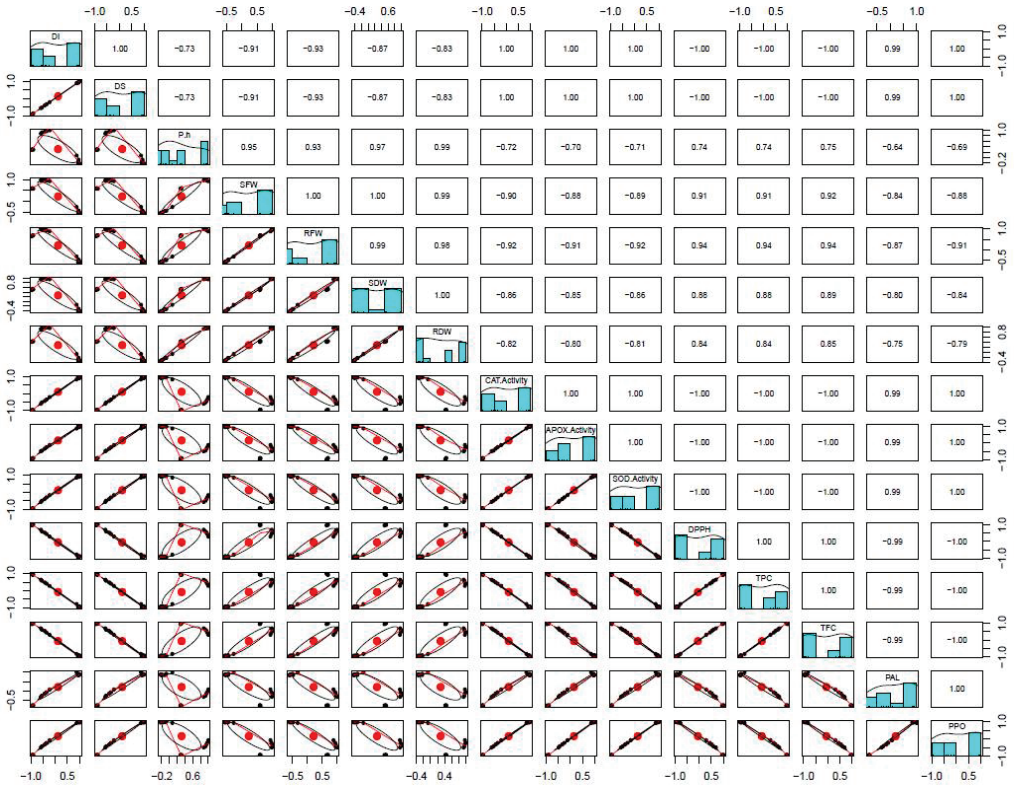

Figure 6. Correlation coefficients (*r*) between different plant parameters. The *r* values from 0.50–0.70 have $p \leq 0.05$ and those from 0.70–1.0 have $p \leq 0.01$.

3.6. Expression Levels of Defense-Related Genes

Pathogenesis-related genes are of great importance in plants that have greatly raised the level of their defense mechanisms against a wide range of pathogens. Therefore, in the third part of the current study, defensin and chitinase gene expression (GE) levels were evaluated. Not surprisingly, both genes showed highly significant levels of expression in the FSEO +P and P treatments, illustrating activation of the defense-related machinery in these treatments. In particular, defensin GE was significantly high (11.84, 11.65, 11.08 and 10.83) for the FSEO +P and P treatments on the second and third day, respectively. On the other hand, significantly low defensin GE levels were seen for other treatments. Accordingly, chitinase GE levels were significantly high (9.13, 9.07, 8.48, 8.26 and 6.29) in the FSEO +P and P treatments on the second and third day, while the other treatments showed relatively low chitinase GE levels (Figure 5).

4. Discussion

The resistance of many crops to fungicides continues to cause serious disease control problems. The practical research experience gathered over the past 50 years has highlighted the importance of using different strategies to control plant diseases. Moreover, scientific research is becoming alarmed not only due to losses from pathogen resistance but also environmental and health concerns. Therefore, there has been increasing interest in the serious pursuit of alternative biological phyto-therapeutic agents. Thus, this study is an attempt toward evaluating the applicability and the potential of essential oils derived from *Foeniculum vulgare* Mill. in the control of *Fusarium* root rot disease in *Vicia faba* L. under greenhouse conditions.

In the present study, we reported the antifungal activity of FSEO against *Fusarium solani* KHA10 in vitro and in vivo. Through GC-MS analysis of oil from *Foeniculum vulgare*, 10 different components were identified. In another study, gas chromatography of essential oils showed the presence of 18 main monoterpenoids in fennel oil. Limonene, trans-anethole, fenchone and estragole were common in fennel oil [21]. Another study reported

that the volatile oil of fennel contains different components: fenchone (1–20%), anethole (40–70%) and estragole (2–9%) [33]. Many other GC-MS screenings enumerated different components: fenchone (1–20%), estragole (2–9%) and anethole (40–70%) [42]. Similarly, in another experiment, fennel essential oil contained fenchone (less than 5%), but bitter types contained 20%. Sweet fennel oil contains 84–90% anethole, but bitter fennel contains 61–70% [18]. In this respect, the fennel seed essential oil, which contained a high amount of menthol, showed good antifungal activity against *F. solani*, as shown in previous results [33].

The results of the current study showed that fennel seed essential oils suppressed mycelium growth of *Fusarium solani* KHA10 in vitro at different concentrations from 25 to 400 µL/mL. Evidently, the inhibition of fungal growth increased with the increase in the concentration of the essential oil [33]. Plant-derived essential oils are compounds that have antibacterial and antifungal activity [15]. An illustration of this is a research study on *Botrytis cinerea*, in which different concentrations of essential oils promisingly and effectively suppressed the growth of *Botrytis cinerea* in a dose-dependent manner [26]. Our revealed data are in high accordance with the findings on another organism [43], which stated that the disease occurrence of powdery mildew on *Zinnia elegans* was significantly diminished through spraying with ginger, cinnamon, fennel and clove essential oils.

Another compelling piece of evidence was found from a research study investigating the high potential antifungal ability of essential oils of *Artemisia indica*, *Mentha spicata*, *Eucalyptus citriodora*, *Cymbopogon citratus* and *Cinnamomum tamala*, recording highly significant activity against *Fusarium oxysporum* and *Aspergillus niger* [44]. Among the tested essential oils, *Cymbopogon citratus* displayed the highest productive antifungal potential against *Fusarium oxysporum* (100% inhibition in mycelial growth) at 40 µL mL^{-1}. *Mentha spicata* showed potent antifungal activity against *Aspergillus niger* (92.93% inhibition in mycelial growth) at 40 µL mL^{-1} concentration [44]. The possible mechanism of action could be attributed to the disruption of the plasma membrane and disorganization of the mitochondrial structure caused by essential oils, as previously reported [45]. Therefore, a recent confirmative hypothesis reported that essential oils contain specific antifungal compounds and fungitoxic agents that inhibit the growth of certain microorganisms [45,46].

The current study reported a significant change in the physiological and morphological characteristics of *Vicia faba* L. plants treated with *F. solani* KHA10, confirming previously reported findings [30], which also stated that the morphological characteristics of asparagus were significantly reduced after inoculation with *Fusarium* species. In this study, the efficacy of fennel seed essential oil was examined in *Vicia faba* L. under greenhouse conditions. Both curative and preventive oil treatments were effective in reducing *F. solani* KHA10 infection. The disease incidence and severity were obviously decreased when plants were treated with FSEO.

Moreover, plant polyphenols are major compounds produced by plants for resistance to pathogens and many other functions [47]. In the present study, total phenolic and flavonoid contents were significantly increased when FSEO was applied on inoculated and non-inoculated faba bean plants when compared with seeds not treated with FSEO and sown in infested soil (pathogen). Similar results were described by [15,34,47], who stated that the phenolic compounds may prevent infection by the pathogen by increasing the mechanical strength of the host's cell walls and thus inhibiting pathogen infection.

Additionally, plant essential oils have unique antioxidant and antimicrobial properties, and are recommended to be a good alternative to synthetic antioxidants and chemical pesticides [48,49]. In this research, inhibition of the fungal activity using essential oil of fennel led to a significant increase in the fresh and dry weight of the shoot and root system of *Vicia faba* L. plants. There was a clear significantly negative relationship (Figure 6) between the degree of disease severity and fresh plant weight, indicating that infection with *F. solani* KHA10 was the main growth-limiting factor in all plants in the present and previous studies [1,25].

In order to evaluate the molecular mechanisms concerned in FSEO-induced resistance in faba bean, the expression of two defense-related genes, defensin and chitinase, was

assessed in treated faba bean at different times. It is worth mentioning here that the expression of defense-related genes can be induced by pathogen inoculation and environmental stresses [33]. The identification of such a broad mechanism involved in defense against pathogens and environmental stresses provides new opportunities for crop improvement. Plant defensins are a family of cysteine-rich peptides, many members of which have been shown to exhibit antimicrobial activity against various microbial attacks [50]. In the present study, we reported the high expression of defensin and chitinase GE treated with the pathogen and FSEO after the third day. These finding show that FSEO may also act as an inducer of the defense-related genes of plants when co-applied with pathogens.

Supporting such findings, it is worth mentioning that essential oils have two advantages: they are safe for use and have a low risk of the microorganisms developing resistance [46]. Essential oils are biodegradable and non-toxic. Since these bioactive compounds are extracted from plants, they are thought to be more acceptable and less risky to the environment than synthetic compounds [10]. In fact, different essential oils as antioxidants are naturally found in plants and have been considered as scavengers of active oxygen [13]. Due to the hydroxyl groups, phenolic compounds play an essential role in their scavenging ability [34,35,50]. Several reports highlighted the rapid advancement of essential oils as biodegradable, less toxic and eco-safe fungi-toxicants, showing the possibilities for their exploitation as natural fungicides [21].

5. Conclusions

Fennel seed essential oil, applied at a concentration of 400 µL/mL, has antifungal activities against *Fusarium solani* KHA10 in vitro and in vivo. The defensin and chitinase gene profile indicated that these genes may play vital roles in the resistance mechanism via reducing *Fusarium* root rot in faba beans. Although more studies are needed to fully verify and understand its mode of action, fennel seed oil is a promising fungicide against *F. solani* KHA10 as well as a plant growth promoter.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10080696/s1, Figure S1: In vitro inhibitory effect of 400 µL/mL of FSEO against *F. solani*, Table S1: Mean Fusarium root rot incidence and severity in pre- and post-emergence damping off after different treatments were applied to *Vicia faba* L. after 3 weeks of treatments, Table S2: Effect of FSEO and F. solani KHA10 on morphological parameters of *Vicia faba* L. under pot conditions at 3 weeks of treatments, Table S3: Biochemical components and antioxidant enzymes of plants, after 6 weeks of treatment.

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Review

Searching for Scientific Explanations for the Uses of Spanish Folk Medicine: A Review on the Case of Mullein (Verbascum, Scrophulariaceae)

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Simple Summary: Mullein (*Verbascum* spp.) has been widely used in Spanish folk medicine to treat several pathologies, and these applications suggest the potential anti-inflammatory action of these plants. Based on the aforementioned, a deep bibliographic review of the chemical composition of the 10 species of Verbascum, catalogued by the Spanish Inventory of Traditional Knowledge related to Biodiversity, and virtual simulations using computer programs were used to demonstrate the molecular evidence supporting the use of these intuitive and traditional popular medicines.

Abstract: *Verbascum* species (common mullein) have been widely used in Spanish folk medicine to treat pathologies related to the musculature, skeleton, and circulatory, digestive, and respiratory systems, as well as to treat infectious diseases and organ-sense illnesses. These applications support the potential anti-inflammatory action of Verbascum phytochemicals. Based on the aforementioned facts, and following a deep bibliographic review of the chemical composition of the 10 species of Verbascum catalogued by the Spanish Inventory of Traditional Knowledge related to Biodiversity, we look for scientific evidences to correlate the traditional medical uses with the chemical components of these plants. To support these findings, in silico simulations were performed to investigate molecular interactions between Verbascum phytochemicals and cellular components. Most of common mullein traditional uses could rely on the anti-inflammatory action of phytochemicals, such as quercetin, and it could explain the employment of these plants to treat a wide range of diseases mediated by inflammatory processes such as respiratory diseases, otitis, arthrosis, and rheumatism among others.

Keywords: Verbascum; traditional knowledge; validation; flavonoid; terpene; inflammatory

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1. Introduction

The genus Verbascum (Scrophulariaceae, Lamiales) comprises more than 300 Eurasiatic species. It is the largest genus of the family, and its origin is the center of the Eastern Mediterranean Basin. In the Iberian Peninsula, it is represented by 26 species [1]. In Spain, they are popularly named "gordolobos" (in English, common mullein), and the Spanish Inventory of Traditional Knowledge related to Biodiversity [2] has catalogued 10 species which have been used to treat a wide range of pathologies. These are *Verbascum pulverulentum* Vill., *V. sinuatum* L., *V. thapsus* L., *V. boerhavii* L., *V. creticum* (L.) Cav., *V. dentifolium* Delile, *V. giganteum* Willk., *V. lychnitis* L., *V. rotundifolium* Ten., and *V. virgatum* Stokes in With.

In order to realize the potential pharmacological application of these species, we must perform a deep analysis of their chemical compositions as a starting point to understand

which phytochemicals could exert the medical actions described in the traditional knowledge. The chemical components of *Verbascum* spp., and the biological actions attributed to these phytochemicals, can be found in the literature [3–17], with the correlation between the phytochemicals' bioactivity and their traditional uses being a key point to validate their traditional ethnobotanical uses.

The aforementioned bibliographic prospection could be complemented by in silico approaches to demonstrate the phytochemicals' affinities using molecular targets. The combination of bibliographic research and computer programming could provide a strong tool to approach the botanical bioactive compounds existing in *Verbascum* spp. with the medical uses collected by folk knowledge.

The objective of this work is to analyze the affinities of phytochemicals from *Verbascum* spp. for mammalian molecular targets to perform a comprehensive scientific validation of its medical uses. This work could support further experimental studies on *Verbascum* spp. extracts and their phytochemicals as therapeutic agents, making the experimental approach easier and eventually contributing to reducing the number of animals employed in pre-clinical testing [18,19].

2. Materials and Methods

2.1. Ethnobotanical Uses and Chemical Composition of Verbascum Used in Spanish Folk Medicine

We first carried out a bibliographic search, looking at the applications recorded by the Spanish Inventory of Traditional Knowledge related to Biodiversity [2], for the 10 *Verbascum* spp. catalogued in the Iberian Peninsula. We summarized them in a table, grouped by diseases and physiological systems.

Afterwards, we performed a bibliographic review of the chemical composition of the 10 Verbascum species. We used the databases Scopus, Dialnet, Medline, PubMed, ScienceDirect, Google Patents, Google Scholar, and Wiley Online. The employed keywords were: "*Verbascum sinuatum*", "*Verbascum thapsus*", "*Verbascum boerhavii*", "*Verbascum creticum*", "*Verbascum dentifolium*", "*Verbascum giganteum*", "*Verbascum lychnitis*", "*Verbascum rotundifolium*", "*Verbascum virgatum*" and/or "activity", "chemical composition", "pharmacology", and "medicin*".

The bibliographic results were managed using a Prisma 2009 Flow Diagram Methodology [20]. A final summary was obtained. It contains the metabolites that had been identified in the aforementioned Verbascum species throughout the published literature and can be consulted in Appendix A (Table A1).

The chemical structures of these metabolites (83 molecules of Table A1) were retrieved from PubChem [21]. This is a database of chemical compounds maintained by the National Centre for Biotechnology Information (NCBI), a branch of the National Library of Medicine of the National Institute of Health (NIH). Structures were drawn and edited using ChemDraw Professional 17.0 (Perkin Elmer, Waltham, MA, USA) and/or Marvin Sketch 19.15 (ChemAxon, Budapets, Hungary). Finally, the respective SMILES codes were also compiled in the abovementioned Table A1 because they are essential to perform the in silico modelling planned for the next stage.

2.2. In Silico Modelling of Verbascum spp. Chemical Constituents' Affinities by Human Molecular Targets

To obtain a virtual prediction of the probable molecular targets of the Verbascum metabolites listed in Table A1, we used the free Software SwissTargetPrediction (STP) [22]. This program allows one to estimate the most probable macromolecular targets of any small molecule assumed to be a bioactive metabolite. The prediction is founded on a combination of 2D and 3D similarity with a library of 370,000 known actives from more than 3000 proteins from 3 species. We focused our predictions on *Homo sapiens* targets. When a metabolite molecule SMILES code is uploaded to the SwissTargetPrediction Website, a document is obtained, which contains a list where proteins are ranked according to the probability of

the protein being a target of the query molecule (phytocompounds). Probabilities of $\geq$0.65 are considered to be significant in the metabolite–protein interaction [22].

We uploaded each of the Verbascum metabolites to the SwissTargetPrediction System; the significant results are summarized in a table available in Appendix B (Table A2). It corresponds to the list of 20 metabolites which showed a significant level of affinity for different targets. The results of Table A2 were analyzed and presented as a frequency histogram figure, structured from the perspective of the STP Target Classes.

The SwissTargetPrediction Program runs with a database system where the proteins included are linked to its own Class Target Classification System.

In summary, the total number of Verbascum metabolites tested in silico was 83, and the metabolites that showed target affinities (finally, 20) were then analyzed, studied, and discussed.

2.3. Comparative Review of Ethnobotanical Uses and Physiopatological Molecular Targets

The discussion consisted of making a qualitative comparison between the traditional use and biological activity of the components. The latter was considered in the published experimental results, which are accessible through bibliographic databases, and the in silico protein affinity tests performed using the aforementioned SwissTargetPrediction Program.

3. Results

3.1. Ethnobotanical Uses and Chemical Composition

The use of *Verbascum* spp. in Spanish traditional medicine includes a wide range of formulations to treat disorders affecting a wide range of systems such as the circulatory, digestive, and respiratory systems, as well as skin diseases, sense organ illnesses, and infectious and parasitic diseases. The main applications collected by the Spanish Inventory of Traditional Knowledge related to Biodiversity [23] for the 10 *Verbascum* spp. catalogued in the Iberian Peninsula are summarized in Table 1, in which we have also included data on the method of administration.

3.1.1. Circulatory System Diseases

Among the circulatory system applications, the anti-hemorrhoidal use of *Verbascum* spp. is the best established, as it has been reported for 7 out of 10 Iberian species. Topical application is the most common posology; it can be accomplished by sitz bath, with the liquid resulting from plant decoction [24–29], or by rubbing the mash or boiled plant onto the affected area [24,30–39]. Rubbing with hairy leaves has also been reported [40–42].

3.1.2. Digestive Apparatus

Digestive system illnesses, in many cases, include conditions caused by an inflammatory process (tooth pain, gumboils, liver and gastric inflammation). Moreover, these species have also been used for their digestive properties and to treat gallstones, diarrhea, and constipation. Again, the liquid resulting after boiling to decoct the plant is the most common posology, together with plant infusions, which are commonly drunk to obtain healing benefits [28,31,36,38,42–49]. Nevertheless, these species can also be used in mouthwashes to treat teeth pain and gumboils [36,38,50,51], or as enemas for constipation, pediatric gut swelling, and indigestion [25]. The topic application of poultices or leaves (boiled or raw) is also used to treat abdominal pain, commonly attributed to liver or gut inflammation or diarrhea [25,28,33–35,52,53].

3.1.3. Respiratory Diseases

The most common way to use *Verbascum* spp., to relieve respiratory system conditions, such as hoarseness, tonsillitis, cold, cough, asthma, or bronchitis, is through the ingestion of a wide variety of preparations (infusions, macerations, syrup) made with common mullein alone or mixed with other plants (mint, rosemary, mallow, hawthorn flower, coltsfoot, thymus and pine leaves, among others) or culinary ingredients (honey and

sugar) [24,25,28,30,31,33,35,36,38–40,43,45,46,49,51,54–68]. The ability of *V. thapsus* extracts to inhibit the growth of bacteria involved in respiratory infections has been proved using antibacterial assays, with the aqueous extracts being the most efficient [69].

3.1.4. Musculature and Skeleton

Regarding the employment of *Verbascum* spp. to treat and relieve conditions affecting the musculature and skeleton, the healing properties attributed to common mullein could rely on its anti-inflammatory action, since most of the conditions treated share a strong inflammatory component (rheumatism, arthritis, swelling, contusions, and broken bones). The formulas employed include fresh, mashed, boiled, or infused plants, and the means of application is topical [25,26,29,30,33,35,43,52,67,70,71].

3.1.5. Skin and Sense Organs

A wide range of skin conditions are treated with *Verbascum* spp., including eczema, exanthema, cysts and zits, insect bites, and nail infections, as well as different types of wounds. The topical application of the liquid, resulting from boiling, infusing, or macerating the plant, is the most common posology [24–26,28,29,31,33,35,36,39,41,42,45–48,52,54,57,60,61,68,72–79]. The species' employment for chilblain relief is another common use (5 out of 10 *Verbascum* spp.). The most common means of application is rubbing the liquid, resulting from decoction [25,27,38,39,43,80–82], which, in Alicante, is carried out in milk instead of water [83]. In Caceres, a lead poultice is applied on the affected area [41].

A liniment made from mullein flowers, boiled or macerated in olive oil, is a common means for treating earache in different parts of Spain (Cataluña, Baleares, and Navarra) [24,25,35,66]. Conjunctivitis is another condition treated with common mullein [24].

3.1.6. Other Uses

Finally, another interesting application of *Verbascum* spp. is the treatment of infectious and parasitic diseases, such as diphtheria, helminthiasis, tuberculosis, typhus, and mange [25,28,35,62,68,77]. Despite the lack of experimental results showing the antimycobacterial action of Verbascum extracts, the British folk knowledge also point to the ability of common mullein to treat tuberculosis. Besides it, the nomenclature and local names of this genus are tightly connected with diseases caused by mycobacteria [84].

3.1.7. Chemical Composition

Spanish *Verbascum* spp. phytocompounds include two main classes: terpenes and flavonoids (see Table A1 and Figure 1). The best characterized species are *V. thapsus* [3,4,6,9,15–17], *V. sinuatum* [10–13], and *V. lychnitis* [5,7,14].

Monoterpene iridoids, sesquiterpenes, triterpene saponins, and phenyl propanoids are isoprene derivatives. Monoterpene iridoids are 10 C terpenes with a cyclopentanopyran cycle. Catalposide and specioside are metabolites belonging to this group. Their chemical structures are very similar, though differing in the way the phenol group is inserted, with specioside being more hydrophobic. Sesquiterpenes are 15 C terpenes, such as buddlindeterpene B. Triterpene saponins (vg. ursolic acid) are 30 C terpenes that reduce the surface tension, easing the mix of lipophilic and hydrophilic phases from liquid substances. Phenilpropanoid alcohols are glycosidic molecules, such as verbascoside and poliumoside.

Flavonoids share a flavonic nucleus (2-phenylbenzopyrane). They have been classified into three subgroups: flavonols, flavones, and O-methylated flavones. Flavones are pheny1-4 benzopyranones, flavonols are 3-hidroxyflavones, and O-metilated flavones have a methyl radical in the 3-hydroxilated part of the main pheny1-4-benzopyranone nucleus. The flavonoid components of Table A1 have a common structure of chromone (1-4 benzopyranone); are characterized by main functional groups such as hydroxyl, and carbonyl; have a conjugated double bond. They are soluble in water and ethanol, and they have oxygen bases varying from moderate to strong.

Some of these components have a powerful physiological activity, which has been shown in several experimental works [85–87]. This activity, usually with a narrow therapeutic margin (little difference between the minimum active concentration and the maximum tolerated concentration), has attracted interest in its associated biochemical processes.

Table 1. Traditional uses of Spanish Verbascum.

	Uses	*Vp*	*Vs*	*Vt*	*Vb*	*Vc*	*Vd*	*Vg*	*Vl*	*Vr*	*Vv*
Circulatory	Anti-hemorrhoidal	B/T	B/T	S/B/T	B/T			T	B	T	
	Leg treatment			B							
	Anti-hypertensive			I/B				I		B	
Digestive	Teeth pain, gumboil	B/T	B	B/T							T
	Digestive	I/B/T		B/T							
	Gastric ulcer/inflammation	B/T	I/B/T	B							
	Liver inflammation	I/B	T	I/B/T				I/B			
	Gallstone	I	I	I/B				I			
	Anti-diarrhoea	T	I	T							
	Constipation			B					E		
Respiratory	Hoarse, tonsillitis	B/T	I/T	I/B/T							
	Cold	B	I	I/B				I			B
	Cough, asthma, bronchitis, hemoptysis	B	I/B/M	I/B			I	I			B
Musculature & Skeleton	Anti-inflammatory (swelling)	B/T		I/B/T							
	Contusion, broken bones	I/T	T	I/B/T							
	Arthrosis, rheumatism		B/T	B/T				I		T	
Skin	Eczema, exanthema	B/T	B/T	T							
	Cysts and zits	T	T	I/B/T				T		T	
	Wounds, ulcers, burns	B/T	M/T	I/B/M/	T			T			
	Horsefly bite			M/T							
	Chilblain	B/T	B	B/T				B/T		B/T	
	Nail conditions			B/T							
Sense	Conjunctivitis	M	M	M							
	Otitis	B/M	M	B							
Infectious parasitic diseases	Diphtheria	T									
	Helminthiasis		B								
	Tuberculosis			I							
	Typhus			T							
	Mange			T							

(*Vp: V. pulverulentum; Vs: V. sinuatum; Vt: V. tapsus; Vb: V. boerhavii; Vc: V. creticum; Vd: V. dentifolium; Vg: V. giganteum; Vl: V. lychnitis; Vr: V. rotundifolium; Vv: V. virgatum).* Administration T: Topic; I: Infusion; B: Boiled; M: Maceration; E: Enem; S: Steam.

3.2. In Silico Modelling of Verbascum spp. Chemical Constituents' Affinities by Human Molecular Targets

The review resulted in a library of 83 molecular structures identified in Verbascum. (Table A1). The application of the SwissTargetPrediction program yielded a final score of 20 molecules with ligand–target interactions with a probability of $\geq$0.65; thus, these were selected, and the rest were discarded. They are summarized in Table 2 and additional data are available in Table A2 (Appendix B).

The chemical structures of the 20 components are plotted in Figure 1, together with the probability values obtained by in silico modelling and target class, according to the SwissTargetPrediction classification.

Figure 2 shows the quantification of cases where the probability is greater than 0.65, in relation to the target class established by SwissTargetPrediction, and shown in Table A2. It

is necessary to emphasize the great affinity for the classes "enzymes" (44 cases), "kinases" (39 cases), and "lyases" (24 cases).

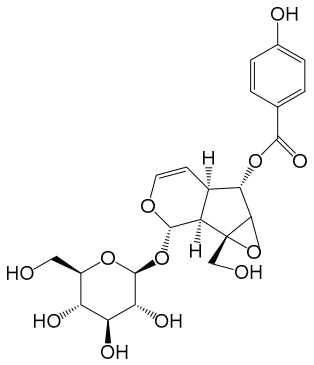
According to the data in Table A2, iridoids (catalposide, specioside) show affinity for the cytosolic protein HSP90AA1 (heat shock protein HSP90-α). The sesquiterpene, buddlindeterpene B, shows affinity for the transcription factors GLI1 and GLI2 (glioma-associated oncogen, which are zinc finger proteins). Ursolic acid, a triterpene saponin, mainly shows affinity for PTPN1 (protein-tyrosine phosphatase 1B) and other phosphatases (PTPN2 or T-cell protein-tyrosine phosphatase, P246666, or low molecular weight phosphotyrosine protein phosphatase), as well as the membrane receptor PTPRF (receptor-type tyrosine-protein phosphatase F), the nuclear receptor RORC (RAR-related orphan receptor γ), the DNA polymerase β (POLB), the aldo-ketoreductase 10 (AKR1B10), and the 11-beta hydroxysteroid dehydrogenase 1 (HSD11B1). The phenylpropanoid glycosides (verbascoside, poliumoside) show affinity for matrix metalloproteinases (MMP2, MMP12). The studied flavones (apigenin, apigenin-7-glucuronide, apigetrin, cynaroside, luteolin, luteolin-7-glucuronide, 6-hydroxyluteolin-7-glucoside, 7-methoxy-luteolin) show a wide profile of affinities, as summarized in Table A2. Among them are affinities for Cit P450, Glyoxalase 1 (GLO1), proinflammatory cytokine IL2, TNF-α secreted proteins, NADPH oxidase (NOX4), and arachinodate lipoxygenase (LOX), and the metalloproteinases (MMP 9 and 12) can be highlighted. The O-metilated flavones (acacetin, acacetin-7-O-α-D-glucoside) show affinity for cytochrome P450 (CYP1B1), interleukin-2 (IL2), and the Tumor Necrosis Factor (TNF-α).

Table 2. Chemical constituents of Spanish Verbascum, which is used in folk medicine, with a ligand–target interaction probability of ≥0.65 calculated by the SwissTargetPrediction software.

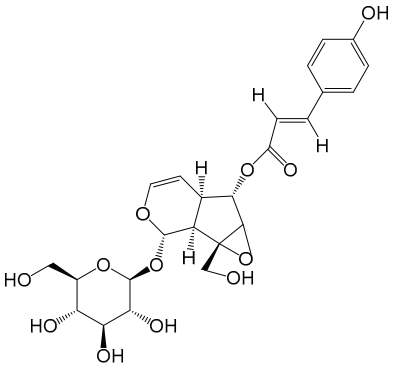
Chemical Group	Component	Species
Monoterpene iridoid	Catalposide	*Vl*
	Specioside	*Vl*
Sesquiterpene	Buddlindeterpene B	*Vt*
Triterpene saponin	Ursolic acid	*Vt*, *Vl*
Phenypropanoid Glycosides	Verbascoside	*Vs*, *Vl*
	Poliumoside	*Vs*, *Vt*, *Vb*
Flavones	Apigenin	*Vt*
	Apigenin-7-glucuronide	*Vt*
	Apigetrin	*Vt*, *Vl*
	Cynaroside	*Vt*, *Vl*
	Luteolin	*Vt*, *Vl*
	Luteolin-7-glucuronide	*Vl*
	6-hydroxyluteolin-7-glucoside	*Vt*, *Vl*
	7-methoxy-luteolin	*Vl*
Flavonol	Quercetin	*Vt*, *Vl*
	3′-methylquercetin	*Vt*, *Vl*
	Kaempferol	*Vt*
	Rutin	*Vt*
O-metilated Flavone	Acacetin	*Vt*
	Acacetin-7-O-α-D-glucoside	*Vt*

Vl = V. lychnitis, Vt = V. thapsus; Vs = V. sinuatum; Vb = V. boerhavii.

Monoterpene Iridoids



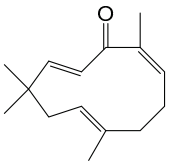
[Catalposide] 0.83 * Cytosolic protein



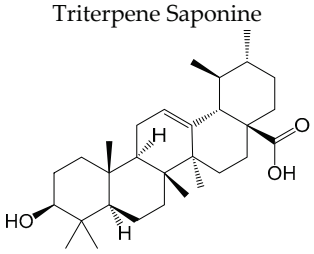
[Specioside] 1.00 * Cytosolic protein

Sesquiterpene

Triterpene Saponine

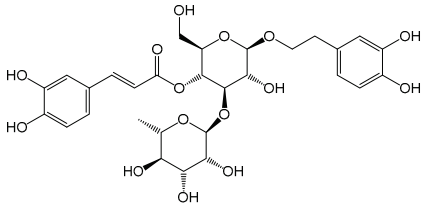


[Buddlindeterpene B] 0.74 * Transcription factor

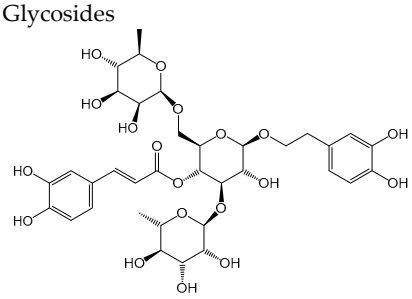


[Ursolic Acid] 0.66–0.95 * Membrane and Nuclear Receptors, Phosphatase and Enzyme.

Phenylpropanoid Glycosides

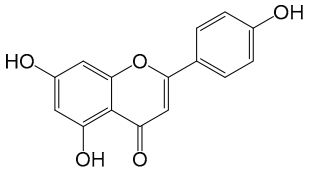


[Verbascoside] 0.74–0.78 * Protease and Kinase

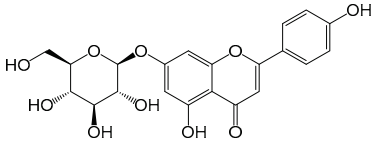


[Poliumoside] 0.68 * Protease

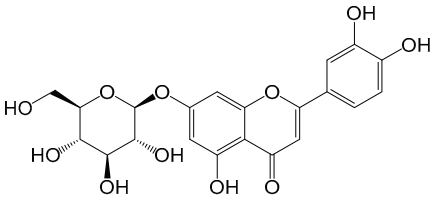
Flavones



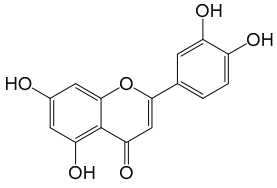
[Apigenin] 1.00 * Enzyme, Secreted protein, Nuclear receptor, Cytochrome P450, Ion channel, Oxidoreductase, Hydrolase, Family A G protein- coupled receptor, Primary active transporter, Kinase and Cytolytic protein.



[Apigetrin] 0.66–1.00 * Secreted protein

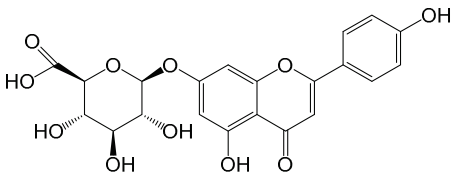


[Cynaroside] 1.00 * Secreted protein

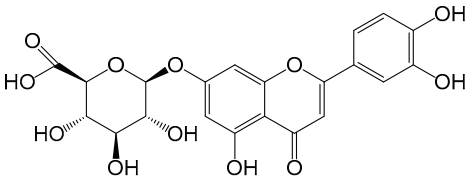


[Luteolin] 0.66–1.00 * Enzyme, Primary active transporter, Phosphatase, Lyase, Secreted protein, Membrane receptor, Oxidoreductase, Isomerase, Protease, Family A G protein-coupled receptor, Kinase, Cytochrome P450 and Cytosolic protein.

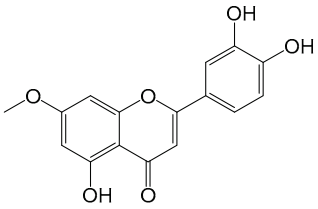
Figure 1. *Cont.*



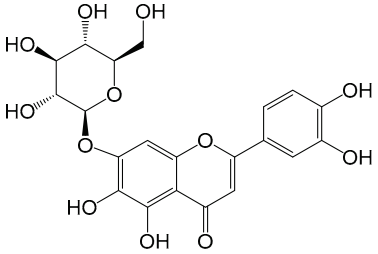
[Apigenin-7-Glucuronide] 1.00 * Enzyme



[Luteolin-7-Glucuronide] 1.00 * Enzyme

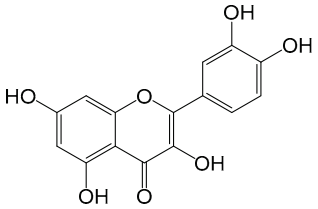


[7-Metoxy-Luteolin] 1.00 * Protease

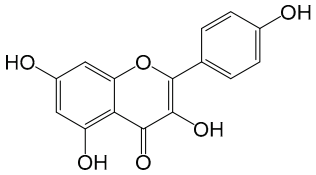


[6-Hydroxyluteolin-7-Glucoside] 1.00 * Secreted protein

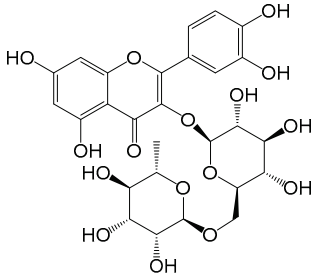
Flavonols



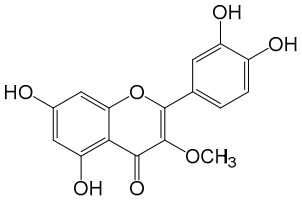
[Quercetin] 0.68–1.00 * Eraser, Kinase, Isomerase, Hydrolase, Enzyme, Family A G protein-coupled receptor, Lyase, Protease, Primary active transporter, Oxidoreductase, and Cytochrome P450.



[Kaempferol] 0.66–1.00 * Protease, Oxidoreductase, Kinase, Enzyme, Hydrolase, Lyase, Family A G protein-coupled receptor, Nuclear receptor, Primary active transporter, Transcription factor and Cytochrome P450.

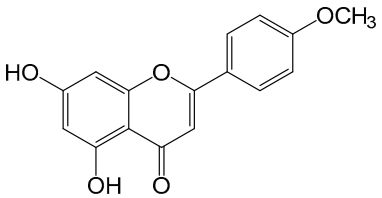


[Rutin] 1.00 * Hydrolase and Family A G protein- coupled receptor

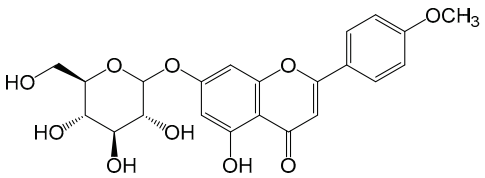


[3-Methylquercetin] 1.00 * Cytochrome P450, Lyase and Oxidorreductase

O-Methylated Flavones



[Acacetin] 1.00 * Cytochrome P450 and secreted protein



[Acacetin-7-O-α-D-glucoside] 1.00 * Secreted protein

Figure 1. Chemical structures of Verbascum components with probability values and target class according to the SwissTargetPrediction classification. * Probability—target class.

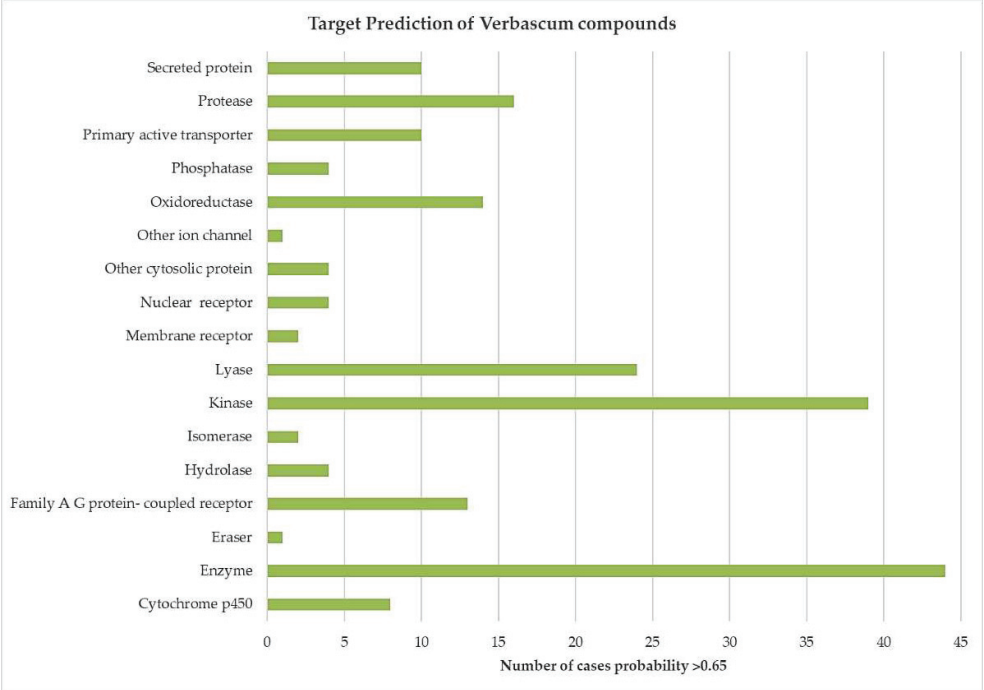


Figure 2. Quantification of cases where the Verbascum molecule–human target affinity is significant in the different classes of targets according to the SwissTargetPrediction classification system.

4. Discussion

4.1. Anti-Inflammatory Action of Verbascum

The role of biological molecules, such as inteleukins (ILs), lipooxygenase (LOX), cyclooxygenase (COX), nuclear factor κB (NF-κB), vascular endothelial growth factor (VEGF), matrix matalloproteinases (MMPs), and tumor necrosis factor (TNF), among others, with the onset of inflammation is well known as well as the link between inflammation and chronic diseases [88]. Therefore, the study of phytochemicals, able to block the action of the aforementioned molecules, is key in the search of new drug candidates to treat chronic diseases and other pathologies with a high inflammatory component.

Most of medicinal applications of *Verbascum* spp. collected from the folk knowledge, have in common an array of inflammatory processes; therefore, understanding the anti-inflammatory molecular mechanisms displayed by Verbascum phytochemicals is essential in order to explain most of its healing properties.

The results generated by our affinities studies show the affinity of flavones (apigenin and luteolin) and flavonols (quercetin, 3′-methylquercetin and kaempferol) by arachinodate-lypoxygenases (LOX), a group of enzymes implicated in the synthesis of eicosanoids, such as leukotriens (LTs), which are molecules with an essential role in cell signaling, being also implicated in inflammation and disorders, such as asthma, skin diseases, rheumatoid arthritis, allergic rhinitis, inflammatory bowel, cardiovascular diseases, cancer, and osteoporosis [89–93]. It is well-known the anti-inflammatory role of polyphenolic compounds [94], in which flavones and flavonols are included. The ability of these compounds to interfere with enzymes implicated in the synthesis of eicosanoids, such as LOX, is one of the molecular mechanisms underlying their anti-inflammatory properties, and the ability of quercetin and lutein to suppress LOX product synthesis has been scientifically proven [90]. Despite our in silico approach cannot provide information about the molecular dynamic of phytochemical-target interaction, the affinity of flavones (apigenin and luteolin) and flavonols (quercetin, 3′-methylquercetin and kaempherol) for LOX, obtained by our in silico approach, is consistent with the scientific results found in the literature, in which the ability of quercetin and luteolin to suppress the formation of LOX products implicated in inflammation, such as LTs, is well demonstrated [90].

The polyphenolic compounds listed in Figure 1 shared a cathechol partial structure, which could be responsible for uncoupling the catalytic cycle of LOX, due to its iron chelating and antioxidant properties [90].

Another interesting result obtained from our in silico studies has shown the affinity of luteolin, quercetin, and kaempferol for interacting with NOX4 (NADPH oxidase-4), an enzyme implicated in the generation of superoxide anions and other downstream reactive oxygen species (ROS) [95]. For example, the protective role of luteolin against inflammation via the NOX4/ROS-NF-κB and MAPK pathways supports our findings and explains the anti-inflammatory action of mullein [96]. Compounds such as acacetin, apigetrin, and cynaroside have a high affinity to interact with the proinflammatory cytokines TNF-α and IL-2, which could also be related to their anti-inflammatory effects. In 2017, a paper from Hu et al. [97] demonstrated the anti-inflammatory effect of the flowers of Chuju (a medical cultivar of *Chrysanthemum morifolim* Ramat), which contain apigetrin and acacetin in their chemical composition [97]. A work of Zhao et al. (2014) [98] showed the ability of acacetin to block T-cell proliferation and IL-2 secretion, both essential to induce the inflammatory response underlying diseases such as rheumatoid arthritis and psoriasis [98]. The anti-inflammatory bioactivity of apigetrin has also been reported in an animal model of acute otitis media [99], which is a traditional use of Verbascum widely reported throughout the Iberian peninsula. Eventually, the anti-inflammatory effect of cynaroside has been demonstrated in a model of human periodontal ligament (hPDL) cells, a cell type essential in the maintenance of the periodontal tissues homeostasis, in which cynaroside has the ability to decrease the expression of pro-inflammatory cytokines, such as TNF-α, induced by LPS treatment [100].

Eventually, the in silico result, showing affinity between ursolic acid and the retinoic acid-related orphan receptor gamma (RORγ), a transcription factor essential for T helper cells differentiation, supported by experimental result showing an effective and selective inhibitory effect of this phytochemical over RORγ, could also explain the anti-inflammatory properties attributed to Verbascum spp. [101].

4.2. Circulatory System Diseases

The most remarkable uses in this section are those related to circulation. The applications of these species against hemorrhoids and varicose veins are related to their local expansion processes in the peripheral circulation. This healing action can be explained by the presence of flavonoids, whose antioxidant and vasodilatory activities are associated with their protective cardiovascular action, widely referred to in the literature [102]. These compounds are common in aqueous extracts from the plants [103], so their presence is expected in many of the preparations recorded in Spanish traditional medicine and listed in Table 1. It has been reported that they are mainly used after being boiled and are then applied externally. The pathologies previously mentioned have also a local inflammatory component, therefore, the anti-inflammatory activity of common mullein, discussed in the previous section, could also underlie this group of healing remedies [93].

The antihypertensive use of *Verbascum* spp. reported in Table 1 could rely on the interaction of Verbascum phytocompounds with the α-adrenergic receptors implicated in peripheral vascular resistance walls. On the one hand, the α-adrenergic antagonist activity of flavonoids could explain Verbascum's antihypertensive action [104]. On the other hand, the affinity of rutin to interact with the α2-adrenoreceptors obtained in our in silico assays, and its anti-hypertensive action reported in the literature [105], could contribute to the antihypertensive action of Verbascum reported from folk knowledge [106].

4.3. Digestive Apparatus

The digestive process begins with activity in the oral cavity, chewing, salivation, and swallowing. Therefore, oral health is essential for proper digestion. The employment of infusions and decoctions, of these plants by Spanish folk medicine, to treat tooth pain and gumboil could be related to the anti-inflammatory activity discussed above. The

anti-inflammatory effect of common mullein could rely on the anti-inflammatory action of its phytochemical cynaroside which has been demonstrated to confer protection against the inflammation underlying the periodontitis [100].

Other applications include for digestive problems, gastric ulcer, or inflammations in different parts of the digestive system (stomach, liver, gallbladder), for which there are treatments described in the traditional Spanish uses of the plant (Table 1). One study indicates the protective effect of ursolic acid against hepatotoxicity in mice [107].

In addition, some of these proteins are specifically related to the physiology of the gastro-intestinal tract. Salivary amylases help to break down food into its molecular components. Parietal cells in the stomach release various acids, pepsins, and enzymes, including gastric amylase, to achieve partial digestion and obtain chemo (semi-fluid and semi-digested mass). Acids also neutralize salivary amylase, favoring gastric intervention. After about an hour, the chimo is pushed into the duodenum, where acidity acquired in the stomach stimulates the release of the hormone secretine. The pancreas then releases hormones, bicarbonate, bile, and numerous pancreatic enzymes, such as lipases (P04054), and those of the lipidic metabolism, such as aldoreductases and most of the ones consigned in the "Enzyme" file of Table A2. These are related to glucose conversion in NADPH-dependent sorbitol, the first step in the poliol pathway of glucose metabolism [108]. Afterwards, thanks to bicarbonate, the acidity of the chimo is changed into an alkaline form, allowing the better degradation of food and also creating a hostile environment for bacteria that survived the passage to the stomach. This process can be carried out effectively and smoothly if the enzyme system is healthy; otherwise, careful supplementation is required [109].

More difficult to validate, however, is the use related to defecation processes. These species have been used as both astringents and laxatives, and the only possible explanation for the traditional use of these plants is that in the first case, diarrhea (for which infusions are taken) has some infectious origin and causes inflammation. In the second case, where enemas are used because of the evacuating effect achieved by the mechanical action of water, this is favored by the presence of triterpene saponins, which have the ability to produce soapy solutions.

4.4. Respiratory Diseases

Respiratory tract pathologies treated with mullein have different etiologies (hoarseness, tonsilitis, colds, coughs, asthma, bronchitis, and even hemoptysis) and treatments, but all have a common feature: the development of inflammatory processes. Besides this, in many cases, fever and cough are displayed. The relief properties of mullein could be explained by its antitussive and expectorant activities, which could be justified by the presence of mucilages in these species [110] which exert demulcent activity [111].

Ursolic acid is one of the most promising substances of biological origin for antimicrobial therapy. It has been identified as a phytochemical inhibitor of the main protease of COVID-19 using molecular modelling approaches [112–114]. Other potential phytochemicals of Verbascum spp., which could be useful to treat COVID-19, are the flavonoids apigenin, luteolin, and quercetin, which have been shown to be replication inhibitors of other coronaviruses [115].

Since, in severe COVID-19 patients, an elevation of pro-inflammatory cytokines occurs, also known as "cytokine storm", that is responsible of deteriorating their health conditions, the search of drugs able block target this "cytokine storm" and suppress the exacerbated inflammatory response is key in the treatment of the complications associated to the disease [116]. Our in silico results have evidenced affinity between mullein phytochemicals (Flavones and O-metilated flavones) and pro-inflammatory cytokines (IL-2 and TNF-α), molecules implicated in inflammatory processes related to the respiratory system and COVID-19 [117–119]. The previously validated anti-inflammatory activity of Verbascum components also supports the potential use of the extracts from the plants tackled in this review to achieve the desire anti-inflammatory action requested to prevent and treat COVID-19 acute clinical profile. The employment of natural compounds with

immunosuppressant properties could be useful as adjuvants to ameliorate the inflammatory process triggered by the out-of-control immune response which could be fatal for the patient, even causing death [120].

Our hypothesis suggesting the employment of Verbascum flavonoids as promising COVID-19 treatment is extensively supported by the existing literature which includes a large number of works using in silico and in vitro approaches which demonstrate the ability of flavonoids to interfere with the viral infection or to prevent/ameliorate the COVID-19 disease effects. Among SARS-CoV2 targets blocked by flavonoids $3CL^{pro}$ (the protease responsible of processing the two polyproteins firstly translated after viral entry) can be highlighted due to its pivotal role in the initiation and progression of the viral cycle and the lack of its human homologue. Apigenin, luteolin, kaempferol, and quercetin are able to inhibit the proteolytic activity of $3CL^{pro}$, quercetin being the most effective. The ability of these phytochemicals to interact with $3Cl^{pro}$ could be due to the ability of the two phenyl groups of flavonoids to interact with the protease substrate binding pocket [121]. Another target is the RNA-dependent RNA polymerase (RdRp) responsible or virus genome replication. The RdRp activity, and therefore the viral replication, is affected by high Zn^{2+} levels and quercetin can act as Zn^{2+} ionophore facilitating the influx of Zn^{2+} into the cell [122]. The last molecular target to deal with SARS-CoV-2 infection is to block the interaction between the SARS-CoV-2 Viral Spike Protein (S) and its cellular receptor, the Angiotensin Converting Enzyme-2 (ACE2) protein, responsible of viral entry. In silico experiments have shown the capacity of two flavonoids (quercetin and luteolin) to block this process [123,124].

A recent review work has studied the potential action mechanisms of Chinese Traditional Medicines to treat COVID-19 by targeting key proteins for the initiation and progression of the disease (ACE 2 and $3CL^{pro}$) or inhibiting inflammatory mediators. The formulas tackled by this review shared components presented in Verbascum spp. such as luteolin, kaempferol and quercetin [125].

The main challenge found in the use of flavonoids, such as quercetin, with a widely supported antiviral action is the poor oral bioavailability due to its reduced absorption and biotransformation during digestion [126,127]. This issue can be tackled through alternative administration ways, such as nasal spray [128] or phytosomes [129].

4.5. Musculature and Skeleton

The use of analgesic, anti-inflammatory, and/or antipyretic drugs is very common in treating a wide range of medical conditions in current clinical pharmacology. Traditional medicine has also used many plants with identical purposes, such as the *Verbascum* spp. studied here. The applications listed in Table 1 extracted from the Spanish National Inventory include a wide spectrum of remedies to treat osteoarthritis, rheumatism, hand crack, kneeache, gout footache, contusions, and even broken bones, all of them characterized by the onset of inflammation and pain. The main aspects considered in the preceding paragraphs have already been discussed within inflammation section.

Pain has been defined by the IASP (International Association for the Study of Pain) as an unpleasant sensory and emotional experience associated with or resembling that associated with actual or potential tissue damage [130]. The phenomenon is a multidimensional entity and nuanced elements of pain are not easy to apprehend when pain is measured with the standard qualitative metrics [130]. From a biochemical and molecular biology point of view, the relationship of certain proteins with painful effects is well known [131], although the potential utility of proteomics to investigate pain management has just started to be considered. Cytochrome P450 [132], gyoxalase I [133], myeloperoxidase [134], and kinases [135] are proteins involved in the physiopathology of pain. Table A2 summarizes how the in silico study points to the great affinity of phytocompounds of these vegetables—particularly quercetin, kaempferol, apigenin, and luteolin—with these proteins.

Osteoarthritis, one of the illnesses treated with common mullein by Spanish traditional medicine, is characterized by the degradation of cartilage, inflammation, and osteophyte

formation in joints. Metalloproteinases are directly related to the onset of this medical condition due to their ability to proteolyze the extracellular matrix [136]. The affinity of some Verbascum phytochemicals (verbascoside, poliumoside, luteolin, quercetin, and kaempferol) for metalloproteinases could explain the traditional employment of mullein in osteoarthritis treatments. This notion is supported by a recent work which suggests the employment of verbascoside to treat osteoarthritis [136]. The employment of an ethanolic extract of Moussonia deppeana (high verbascoside content) shows an anti-edematous action in an experimental model of arthritis [137]. The ability of quercetin to reduce the severity of rheumatoid arthritis has also been demonstrated in vivo [138]. Another molecular mechanism, implicated in rheumatoid arthritis, is the invasion of fibroblast-like synoviocytes (FLS), which is responsible for cartilage destruction. Again, the metalloproteinases are involved in FLS invasion and kaempferol is able to reduce FLS migration and invasion both in vitro and in vivo [139].

A similar reasoning can be found regarding fever. Antithermic action is related to TNF-α secreted proteins [140] (P01375, Table A2), which have shown an in silico affinity with Verbascum flavones (6-hydroxyluteolin-7-glucoside, apigetrin, and cynaroside) and O-metilated flavones, such as acacetin-7-O-α-D-glucoside.

4.6. Skin and Sense Organs

The topical dermatological use of various extracts (infusion, boiling, maceration) from these plants for the treatment of occasional or repetitive local eruptions (cysts, zits, eczemas, exanthemas), accidental or more serious conditions (wounds, ulcers, burns, bites), and even eye or ear inflammations are justified by their anti-inflammatory power reported throughout this manuscript.

The employment of common mullein to treat otitis could be explained by the presence of apigetrin in its chemical composition. We have shown the high affinity of apigetrin for TNF-α and IL-2 (P01375 and P60558, respectively), both belonging to the cytokine family and implicated in inflammatory processes. This hypothesis is supported by a recent work which demonstrates the healing effect of apigetrin in otitis media due to its ability to suppress inflammation and oxidative stress. Treatment with apigetrin reduces mucosa thickness, inhibits the inflammatory response by downregulating neutrophils and macrophages, and reduces ROS generation, eventually alleviating otitis [99].

4.7. Other Uses

Other popular uses, such as in the treatment of infectious diseases and parasitosis (diphtheria, helminthiasis, tuberculosis, typhus, and mange), require a direct validation that is difficult to explain with the data currently available. Indirectly, all the anti-inflammatory actions discussed throughout this work need to be taken into consideration.

5. Conclusions

The use of Spanish Verbascum spp. is in traditional medicine as a healing plant related to various pathologies, most of them involving inflammatory processes, can be justified from a scientific point of view, based on the chemical composition of these plants and the biological activities tested in vitro or in vivo employing the isolated phytochemicals or the plant extract itself, which can be found through the large bibliographic databases surveyed. The bibliographic prospection is supported by a simple in silico approach to look for specific protein affinities, in order to conduct the aforesaid bibliographic search.

The popular and most common use of Verbascum spp. is linked to its anti-inflammatory properties, which could be explained by the presence of flavonoids such as luteolin, quercetin, apigenin, and kaempferol within chemical composition. The anti-inflammatory properties of these molecules are well validated in the literature. Our in silico study's findings are in line with the experimental results found in the existing bibliography and have allowed us to select the phytochemicals with potential biological activities, among the preliminary list of compounds. This approach validates the employment of simple in silico

studies aimed to obtain the molecule-target affinities as a useful tool to be employed before starting bibliographic or experimental works aimed to validate the biological activities of phytochemicals. This kind of studies have a pivotal role to underlie the search of potential pharmacological compounds to be used as drug candidates to treat a wide range of pathologies. In the case of the species studied, the activity of molecules such as the flavonoids (apigenin, apigetrin, cynaroside, luteolin, quercetin, kaempferol, rutin, acacetin), iridoids (catalposide, specioside), phenylpropanoids (verbascoside, poliumoside), sesquiterpenes (buddlindeterpene), and saponins (ursolic acid) could serve as inspiration for the design of improved drugs to treat a wide range of pathologies, including respiratory pathologies, which are of particular interest at the moment, in the context of the COVID 19 pandemic.

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Abbreviations

Vp	*Verbascum pulverulentum*
Vs	*V. sinuatum*
Vt	*V. tapsus*
Vb	*V. boerhavii*
Vc	*V. creticum*
Vd	*V. dentifolium*
Vg	*V. giganteum*
Vl	*V. lychnitis*
Vr	*V. rotundifolium*
Vv	*V. virgatum*
SMILES	Simplified Molecular Input Line Entry Specification
IASP	International Association for the Study of Pain

Appendix A

Table A1. Metabolites of Spanish Verbascum (*Vp: V. pulverulentum; Vs: V. sinuatum; Vt: V. thapsus; Vb: V. boerhavii; Vc: V. creticum; Vd: V. dentifolium; Vg: V. giganteum; Vl: V. lychnitis; Vr: V. rotundifolium; Vv: V. virgatum*) and SMILES code.

	Metabolite	Species	Reference	SMILES Code
			Monoterpene Iridioids	
1	Aucubin	*Vs*, *Vt*, *Vl*, *Vv*	[6,9,17]	C1=COC(C2C1C(C=C2CO)O)OC3C(C(C(C(O3)CO)O)O)O
2	6-O-β-D-glucopyranosyl aucubin	*Vs*	[9]	[H][C@@]23C=CO[C@@H](OC1[C@H](O)O[C@H](CO)[C@@H](O)[C@@H]1O)[C@]2([H])C(CO)=C[C@H]3O[C@H]4[C@H](O)O[C@H](CO)[C@@H](O)[C@@H]4O
3	Sinuatol	*Vs*	[9,11]	[H][C@@]34C=COC(O[C@H]2O[C@](CO)(O[C@@H]1O[C@@H](C)[C@H](O)[C@@H](O)[C@H]1O)[C@@H](O)[C@H](O)[C@H]2O)[C@]3([H])C(CO)=CC4
4	6-O-β-D-xylopyranosyl aucubin	*Vs*, *Vt*	[6,9,10,17]	[H][C@@]23C=CO[C@@H](O[C@H]1[C@H](O)O[C@H](CO)[C@@H](O)[C@@H]1O)[C@]2([H])C(CO)=C[C@H]3O[C@H]4[C@@H](O)O[C@H](CO)[C@@H]4O
5	6-O-α-L-sinuatosyl aucubin	*Vs*	[9,12]	[H][C@@]23C=CO[C@@H](O[C@H]1O[C@@H](CO)[C@H](O)[C@@H](O)[C@@H]1O)[C@]2([H])C(CO)=C[C@H]3OC5O[C@@H](CO)[C@H](O)[C@@H](OC4OCC(O)C(O)C4O)[C@@H]5O
6	Sinuatoside	*Vs*	[9,13]	OCC4=C[C@@H](OC2OC(CO)C(O)C(OC1OCC(O)C(O)C1O)C2O)C5C=CO[C@@H](OC3OC(CO)C(O)C(O)C3O)C45
7	Aucuboside	*Vs*, *Vl*	[9,13]	C1=COC(C2C1C(C=C2CO)O)OC3C(C(C(C(O3)CO)O)O)O
8	Catalpol	*Vs*, *Vt*, *Vl*	[6,9,17]	C1=COC(C2C1C(C3C2(O3)CO)O)OC4C(C(C(C(O4)CO)O)O)O
9	Isocatalpol	*Vt*, *Vl*	[6,9,17]	[H][C@@]24C=CO[C@@H](OC1OC(CO)C(O)[C@H](O)C1O)[C@]2([H])[C@@]3(CO)O[C@H]3[C@H]4O
10	Methylcatalpol	*Vt*, *Vl*	[6,9,17]	CO[C@@H]1[C@@H]2O[C@]2(CO)[C@H]2[C@H](O[C@@H]3O[C@H](CO)[C@@H](O)[C@H](O)[C@H]3O)OC=C[C@@H]12
11	6-O-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	CC6O[C@H](O[C@H]3C2C=CO[C@@H](OC1OC(CO)C(O)[C@@H](O)[C@H]1O)C2[C@]4(O)O[C@H]34)C(COC(=O)C=Cc5ccccc5)C(O)[C@@H]6O
12	Saccatoside	*Vt*, *Vv*	[6,9,17]	C[C@H]1[C@@H]([C@H]([C@H]([C@@H](O1)O[C@H]2[C@@H]3C=CO[C@H]([C@@H]3[C@@]4([C@H]2O4)CO)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O)O)OC(=O)/C=C/C6=CC=C(C=C6)O)O)O
13	6-O-(3″-O-p-coumaroyl)-α-Lrhamnopyranosylcatalpol	*Vs*, *Vt*, *Vv*	[6,9,17]	C[C@@H]6O[C@@H](O[C@@H]3C2C=CO[C@H](O[C@@H]1OC(CO)[C@H](O)C(O)[C@@H]1O)C2[C@@]4(CO)O[C@@H]34)C(O)C(OC(=O)C=Cc5cccc(O)cc5)[C@@H]6O
14	6-O-(4″-O-p-coumaroyl)-α-Lrhamnopyranosylcatalpol	*Vt*	[6,9,17]	[H][C@@]24C=CO[C@@H](O[C@@H]1OC(CO)[C@H](O)[C@H](O)C1O)[C@]2([H])[C@@]3(CO)O[C@H]3[C@H]4O[C@H]5O[C@@H](C)C(O)C(O)C5OC(=O)C=Cc6ccc(O)cc6
15	6-O-(2″-O-(p-methoxy-trans-cinnamoyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6ccc(C=CC(=O)OC1C(O)[C@H](O)[C@H](C)O[C@@H]1O[C@H]4C3C=CO[C@@H](O[C@H]2OC(CO)[C@@H](O)C(O)[C@H]2O)C3[C@]5(CO)O[C@H]45)cc6
16	6-O-(3″-O-(p-methoxy-trans-cinnamoyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6ccc(C=CC(=O)OC5[C@H](O)[C@H](C)O[C@H](O[C@H]3C2C=CO[C@@H](O[C@H]1OC(CO)[C@@H](O)C(O)[C@H]1O)C2[C@]4(CO)O[C@H]34)C5O)cc6
17	Verbascoside A	*Vt*	[6,9,17]	C[C@H]1[C@@H]([C@H]([C@H]([C@@H](O1)O[C@H]2[C@@H]3C=CO[C@H]([C@@H]3[C@@]4([C@H]2O4)CO)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O)O)O)O)OC(=O)/C=C/C6=CC=C(C=C6)OC
18	6-O-[2″-O-(3,4-dihydroxy-trans-cinnamoyl)]-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	C=C(C=Cc1ccc(O)c(O)c1)OC2C(O)[C@H](O)[C@H](C)O[C@@H]2O[C@H]5C4C=CO[C@@H](O[C@H]3OC(CO)[C@@H](O)C(O)[C@H]3O)C4[C@]6(CO)O[C@H]56
19	6-O-[4″-O-(3,4-dihydroxy-trans-cinnamoyl)]-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	C=C(C=Cc1ccc(O)c(O)c1)O[C@@H]6[C@H](C)O[C@H](O[C@H]4C3C=CO[C@@H](O[C@H]2OC(CO)[C@@H](O)C(O)[C@H]2O)C3[C@]5(CO)O[C@H]45)C(O)C6O
20	6-O-[3″-O-(3,4-dimethoxy-trans-cinnamoyl)]-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	C=C(C=Cc1ccc(OC)c(OC)c1)OC6[C@H](O)[C@H](C)O[C@H](O[C@H]4C3C=CO[C@@H](O[C@H]2OC(CO)[C@@H](O)C(O)[C@H]2O)C3[C@]5(CO)O[C@H]45)C6O

Table A1. *Cont.*

	Metabolite	Species	Reference	SMILES Code
21	6-O-(2″-O-feruloyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6cc(C=CC(=O)OC1C(O)[C@H](O)[C@H](C)O[C@@H]1O[C@H]4C3C=CO[C@@H](O[C@H]2OC(CO)[C@@H](O)C(O)[C@H]2O)C3[C@]5(CO)O[C@H]45)ccc6O
22	6-O-(4″-O-feruloyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6cc(C=CC(=O)O[C@@H]5[C@H](C)O[C@H](O[C@H]3C2C=CO[C@@H](O[C@H]1OC(CO)[C@@H](O)C(O)[C@H]1O)C2[C@]4(CO)O[C@H]34)C(O)C5O)ccc6O
23	6-O-(2″-O-isoferuloyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6ccc(C=CC(=O)OC1C(O)[C@H](O)[C@H](C)O[C@@H]1O[C@H]4C3C=CO[C@@H](O[C@H]2OC(CO)[C@@H](O)C(O)[C@H]2O)C3[C@]5(CO)O[C@H]45)cc6O
24	6-O-(3″-O-isoferuloyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6ccc(C=CC(=O)OC5[C@H](O)[C@H](C)O[C@H](O[C@H]3C2C=CO[C@@H](O[C@H]1OC(CO)[C@@H](O)C(O)[C@H]1O)C2[C@]4(CO)O[C@H]34)C5O)cc6O
25	6-O-(4″-O-isoferuloyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6ccc(C=CC(=O)O[C@@H]5[C@H](C)O[C@H](O[C@H]3C2C=CO[C@@H](O[C@H]1OC(CO)[C@@H](O)C(O)[C@H]1O)C2[C@]4(CO)O[C@H]34)C(O)C5O)cc6O
26	Pulverulentoside I	*Vp*, *Vs*, *Vt*	[6,9,17]	C=C(C=Cc1ccc(OC)cc1)OC6[C@@H](O[C@H]4C3C=CO[C@@H](O[C@H]2OC(CO)[C@@H](O)C(O)[C@H]2O)C3[C@]5(CO)O[C@H]45)O[C@@H](C)[C@@H](O)C6OOC(C)=O
27	6-O-(2″-O-p-methoxy-trans-cinnamoyl-4″-O-asetyl)-α-L-rhamnopyranosylcatalpol	*Vt*	[6,9,17]	COc6ccc(C=CC(=O)OC1C(O)[C@H](OC(C)=O)[C@H](C)O[C@@H]1O[C@H]4C3C=CO[C@@H](O[C@H]2OC(CO)[C@@H](O)C(O)[C@H]2O)C3[C@]5(CO)O[C@H]45)cc6
28	Pulverulentoside II	*Vp*	[9]	COc6ccc(C=CC(=O)OC5C(O)C(C)OC(OC3C2C=COC(OC1OC(CO)C(O)C(O)C1O)C2C4(CO)OC34)C5OC(C)=O)cc6O
29	Catalposide	*Vl*	[5,9]	C1=COC(C2C1C(C3C2(O3)CO)OC(=O)C4=CC=C(C=C4)O)OC5C(C(C(C(O5)CO)O)O)O
30	Specioside	*Vl*	[4,83]	C1C(C(=CC(=O)OC2=C1C=CC(=C2)OC3C(C(C(C(O3)CO)O)O)O)C4=CC=C(C=C4)O)O.C1C(C(=CC(=O)OC2=C1C=CC(=C2)O)C3=CC=C(C=C3)O)OC4C(C(C(C(O4)CO)O)O)O
31	Ajugol	*Vt*, *Vv*	[6,9,17]	CC1(CC(C2C1C(OC=C2)OC3C(C(C(C(O3)CO)O)O)O)O)O
32	6-O-benzoyl ajugol	*Vt*	[6,9,17]	[H][C@@]23C=CO[C@@H](O[C@@H]1O[C@H](CO)[C@@H](O)[C@H](O)[C@H]1O)[C@]2([H])[C@@](C)(O)C[C@H]3OC(=O)c4ccccc4
33	6-O-syringoyl ajugol	*Vt*	[6,9,17]	CC1(CC(C2C1C(OC=C2)OC3C(C(C(C(O3)CO)O)O)O)O)OC(=O)C4=CC(=C(C(=C4)OC)O)OC)O
34	6-O-vanilloyl ajugol	*Vt*	[6,9,17]	CC1(CC(C2C1C(OC=C2)OC3C(C(C(C(O3)CO)O)O)O)O)OC(=O)C4=CC(=C(C=C4)O)OC)O
35	Harpagide	*Vs*, *Vt*	[6,9,17]	CC1(CC(C2(C1C(OC=C2)OC3C(C(C(C(O3)CO)O)O)O)O)O)O
36	Harpagoside	*Vp*, *Vs*, *Vt*	[6,9,17]	CC1(CC(C2(C1C(OC=C2)OC3C(C(C(C(O3)CO)O)O)O)O)O)OC(=O)C=CC4=CC=CC=C4
37	Lychnitoside	*Vl*	[9]	OCC2=CO[C@@H](O[C@@H]1O[C@H](CO)[C@@H](O)[C@H](O)[C@H]1O)C3C=CCC23
38	Lateroside	*Vt*	[8]	[H][C@@]24C=CO[C@@H](O[C@@H]1O[C@H](CO)[C@@H](O)[C@H](O)[C@H]1O)[C@]2([H])[C@@](C)(OC(=O)C=Cc3ccccc3)C[C@H]4O
39	5-O-α-L-rhamnopyranosy (1α-3)-[α-D-glucuronopyranosyl (1α-6)]-α-D-glucopyranoside	*Vt*	[8]	CC8OC(OC1C(O)C(O)C(O)OC1OC2C(O)C(O)C(C(=O)O)OC2OC7CC[C@]6(C)C5CC=C4C3CC(C)(C)CC(O)[C@]3(C)CC[C@@]4(C)[C@]5(C)CCC6C7(C)CO)C(O)C(O)C8O
40	Ningpogenin	*Vt*	[8]	[H][C@]12C=C(CO)[C@@H](CO)[C@@]1([H])CC(=C)O2
41	10-deoxyeucommiol	*Vt*	[8]	CC1=C(CO)C(CCO)[C@@H](O)C1
42	Jioglutolide	*Vt*	[8]	C[C@@]1(C[C@H]([C@H]2[C@@H]1COC(=O)C2)O)O
43	6-β-hydroxy-2-oxabicyclo [4.3.0]Δ8-9-nonen-1-one	*Vt*	[8]	[H][C@@]12CCOC(=O)C1=C(C)C[C@H]2O
44	8-cinnamoylmyoporoside	*Vt*	[8]	C[C@@]1(C[C@H](C2[C@@H]1[C@@H](OC=C2)O[C@H]3[C@@H]([C@H]([C@@H]([C@H](O3)CO)O)O)O)O)OC(=O)/C=C/C4=CC=CC=C4
45	Verbthasin A	*Vt*	[8]	[H][C@@]12COC(=C)[C@]1([H])C[C@@H](O)C2=CCO

Table A1. *Cont.*

	Metabolite	Species	Reference	SMILES Code
			SESQUITERPENES	
46	Buddlindeterpene A	*Vt*	[8]	CC2=CCC[C@]1(C)O[C@@H]1CC(C)(C)C=CC2=O
47	Buddlindeterpene B	*Vt*	[8]	CC1=CCC(C)(C)C=CC(=O)C(C)=CCC1
48	Buddlindeterpene C	*Vt*	[8]	C=C[C@@]1(C)CCC2C(O)(C1=O)[C@H]4CC3[C@@](C)(C)CCC[C@]23CO4
			Triterpene Saponines	
49	Thapsuine B	*Vt*, *Vl*	[3,4,9]	[H][C@@]49C=C[C@]23OC[C@@]1(CCC(C)(C)C[C@]12[H])CC[C@@]3(C)[C@]4(C)CC[C@]%10([H])[C@](C)(CO)C(O[C@H]8OC(COC6OC(C)C(OC5OC(CO)C(O)C(O)C5O)C(O)C6O)[C@@H](O)C(OC7CC(C)C(O)C(O)C7O)[C@H]8O)CC[C@]9%10C
50	Hydroxythapsuine B	*Vt*	[3,4,9]	[H][C@@]49C=C[C@]23OC[C@@]1(CCC(C)(O)C[C@]12[H])CC[C@@]3(C)[C@]4(C)CC[C@]%10([H])[C@](C)(CO)C(O[C@H]8OC(COC6OC(C)C(OC5OC(CO)C(O)C(O)C5O)C(O)C6O)[C@@H](O)C(OC7CC(C)C(O)C(O)C7O)[C@H]8O)CC[C@]9%10C
51	Saikogenin A	*Vt*	[3,4,9]	CC1(CCC2(C(CC3(C(=C2C1)C=CC4C3(CCC5C4(CCC(C5(C)CO)O)C)C)C)O)CO)C
52	Thapsuine A	*Vt*, *Vl*	[3,4,9]	CC1C(C(C(C(O1)OC2C(C(OC(C2O)OC3CCC4(C(C3(C)CO)CCC5(C4C=CC67C5(CCC8(C6CC(CC8)(C)C)CO7)C)C)C)COC9C(C(C(C(O9)C)OC1C(C(C(C(O1)CO)O)O)O)O)O)O)O)O)O
53	Hydroxythapsuine A	*Vt*	[3,4,9]	CC%10OC(OC9C(O)C(COC2OC(C)C(OC1OC(CO)C(O)C(O)C1O)C(O)C2O)OC(OC8CC[C@]7(C)[C@H]6C=C[C@]45OC[C@@]3(CCC(C)(O)C[C@H]34)CC[C@@]5(C)[C@]6(C)CC[C@H]7[C@]8(C)CO)C9O)C(O)C(O)C%10O
54	Ursolic acid	*Vt*, *Vl*	[8]	CC1CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C)C)O)C)C)C2C1C)C)C(=O)O
55	Veratric acid	*Vt*	[8]	COC1=C(C=C(C=C1)C(=O)O)OC
56	β-spinasterol	*Vt*	[8]	[H]C(=C([H])C(CC)C(C)C)[C@@H](C)[C@@]4([H])CC[C@@]3([H])C2=CC[C@@]1([H])C[C@@H](O)CC[C@]1(C)[C@@]2([H])CC[C@@]34C
57	Hydroxythapsuine	*Vt*	[9]	[H][C@@]49C=C[C@]23OC[C@@]1(CCC(C)(O)C[C@]12[H])CC[C@@]3(C)[C@]4(C)CC[C@]%10([H])[C@](C)(CO)C(O[C@H]8OC(COC6OC(C)C(OC5OC(CO)C(O)C(O)C5O)C(O)C6O)[C@@H](O)C(OC7CC(C)C(O)C(O)C7O)[C@H]8O)CC[C@]9%10C
58	3-O-fucopyranosyl saikogenin F	*Vt*	[9]	C[C@@H]7O[C@H](OC6CC[C@]5(C)C4C=C[C@]23OC[C@@]1(CC[C@](C)(C)CC12)[C@@H](O)C[C@@]3(C)[C@]4(C)CCC5[C@]6(C)O)[C@@H](O)[C@H](O)[C@@H]7O
			Phenilpropanoid Glycosides	
59	Verbascoside (=acetoside)	*Vs*, *Vl*	[9,16]	CC1C(C(C(C(O1)OC2C(C(OC(C2OC(=O)C=CC3=CC(=C(C=C3)O)O)CO)OCCC4=CC(=C(C=C4)O)O)O)O)O)O
60	Poliumoside	*Vs*, *Vt*, *Vb*	[9]	CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OCCC3=CC(=C(C=C3)O)O)O)OC4C(C(C(C(O4)C)O)O)O)OC(=O)C=CC5=CC(=C(C=C5)O)O)O)O)O
61	Forsythoside B	*Vt*, *Vl*	[9]	CC1C(C(C(C(O1)OC2C(C(OC(C2OC(=O)C=CC3=CC(=C(C=C3)O)O)COC4C(C(CO4)(CO)O)O)OCCC5=CC(=C(C=C5)O)O)O)O)O)O
62	Arenarioside	*Vt*	[9]	CC1C(C(C(C(O1)OC2C(C(OC(C2OC(=O)C=CC3=CC(=C(C=C3)O)O)COC4C(C(C(CO4)O)O)O)OCCC5=CC(=C(C=C5)O)O)O)O)O)O
63	Alyssonoside	*Vt*	[9]	CC1C(C(C(C(O1)OC2C(C(OC(C2OC(=O)/C=C/C3=CC(=C(C=C3)O)OC)COC4C(C(CO4)(CO)O)O)OCCC5=CC(=C(C=C5)O)O)O)O)O)O
64	Leucosceptoside B	*Vt*	[9]	[H][C@@]5(O[C@@H]3C[C@H](OCCc1ccc(OC)c(O)c1)O[C@H](CO[C@@H]2OC[C@](O)(CO)[C@H]2O)[C@H]3OC(=C)C=Cc4ccc(O)c(OC)c4)O[C@@H](C)[C@H](O)[C@@H](O)[C@H]5O
65	Cistanoside B	*Vt*	[9]	CC1C(C(C(C(O1)OC2C(C(OC(C2OC(=O)/C=C/C3=CC(=C(C=C3)O)OC)COC4C(C(C(C(O4)CO)O)O)O)OCCC5=CC(=C(C=C5)O)OC)O)O)O)O
			Flavones	
66	Apigenin	*Vl*	[8]	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O
67	Apigenin-7-glucuronide	*Vl*	[8]	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)C(=O)O)O)O)O)O)O
68	Luteolin	*Vt*, *Vl*	[9,15]	C1=CC(=C(C=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O)O

Table A1. *Cont.*

	Metabolite	Species	Reference	SMILES Code
69	Luteolin-5-glucoside	*Vl*	[8]	C1=CC(=C(C=C1C2=CC(=O)C3=C(O2)C=C(C=C3OC4C(C(C(C(O4)CO)O)O)O)O)O)O
70	Luteolin-7-glucuronide	*Vl*	[8]	C1=CC(=C(C=C1C2=CC(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)C(=O)O)O)O)O)O)O)O
71	7-methoxy-luteolin	*Vl*	[8]	COc3cc(O)c2c(=O)cc(c1ccc(O)c(O)c1)oc2c3
			Triterpene Saponines	
72	Cynaroside	*Vt*	[9]	C1=CC(=C(C=C1C2=CC(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)CO)O)O)O)O)O)O
73	Apigetrin	*Vt*	[9]	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)CO)O)O)O)O)O
74	4′,7-dihydroxyflavone-4′-rhamnoside	*Vt*	[9]	C[C@H]4O[C@H](Oc3ccc(c2cc(=O)c1ccc(O)cc1o2)cc3)[C@@H](O)[C@@H](O)[C@@H]4O
75	6-hydroxyluteolin-7-glucoside	*Vt*	[9]	C1=CC(=C(C=C1C2=CC(=O)C3=C(C(=C(C=C3O2)OC4C(C(C(C(O4)CO)O)O)O)O)O)O)O
			Flavonols	
76	Quercetin	*Vt*, *Vl*	[9]	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O
77	Quercetin-7-glucuronide	*Vl*	[8]	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)C(=O)O)O)O)O)O)O)O)O
78	3′-methylquercetin	*Vt*	[9]	COC1=C(C=CC(=C1)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O
79	Kaempferol	*Vt*	[9]	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O
80	Rutin	*Vt*	[9]	CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OC3=C(OC4=CC(=CC(=C4C3=O)O)O)C5=CC(=C(C=C5)O)O)O)O)O)O)O)O)O
			O-Metilated Flavones	
81	Acacetin	*Vl*	[9]	COC1=CC=C(C=C1)C2=CC(=O)C3=C(C=C(C=C3O2)O)O
82	Acacetin-7-O-α-D-glucoside	*Vt*	[8]	O=c3cc(c1ccc(O)c(O)c1)oc4cc(OC2OC(CO)C(O)C(O)C2O)cc(O)c34
83	Patuletin	*Vl*	[8,9]	COC1=C(C2=C(C=C1O)OC(=C(C2=O)O)C3=CC(=C(C=C3)O)O)O

Appendix B

Table A2. Targets and metabolites of *Verbascum* spp. Probability calculated by SwissTargetPrediction (http://www.swisstargetprediction.ch/ accessed date 10 June 2020).

Target Class	Target	Uniprot ID	Metabolite	Probability	Chemical Group
Cytochrome P450	Cytochrome P450 19A1	P11511	Apigenin	1.00	Flavone
			Quercetin	1.00	Flavonol
	Cytochrome P450 1B1	Q16678	3′-methylquercetin	1.00	Flavonol
			Kaempferol	1.00	
			Quercetin	1.00	
			Acacetin	1.00	O-metilated Flavone
			Apigenin	1.00	Flavone
			Luteolin	1.00	
Enzyme	11-beta- hydroxysteroid dehydrogenase 1	P28845	Ursolic acid	0.66	Triterpene
	Aldehyde reductase (by homology)	P14550	Quercetin	0.66	Flavonol
	Aldo-keto reductase family 1 member B10	O60218	Apigenin	0.68	Flavone
			Luteolin	0.68	
			Ursolic acid	0.66	Triterpene
	Aldo-keto reductase family 1 member C1 (by homology)	Q04828	Quercetin	0.70	Flavonol
	Aldo-keto reductase family 1 member C2 (by homology)	P52895	Quercetin	0.73	Flavonol

Table A2. *Cont.*

Target Class	Target	Uniprot ID	Metabolite	Probability	Chemical Group
	Aldo-keto reductase family 1 member C4 (by homology)	P17516	Quercetin	0.80	Flavonol
	Aldo-keto-reductase family 1 member C3 (by homology)	P42330	Quercetin	0.80	Flavonol
	Aldose reductase	P15121	Luteolin	1.00	Flavone
			Apigenin	1.00	
			Apigenin-7-glucuronide	1.00	
			7-methoxy-luteolin	1.00	
			Luteolin-7-glucuronide	1.00	
			Quercetin	1.00	Flavonol
			Kaempferol	1.00	
	Arachidonate 12-lipoxygenase	P18054	Quercetin	1.00	Flavonol
	Arachidonate 15-lipoxygenase	P16050	Quercetin	1.00	Flavonol
	Arginase-1 (by homology)	P05089	Luteolin	1.00	Flavone
	DNA polymerase beta	P06746	Ursolic acid	1.00	Triterpene
	DNA-(apurinic or apyrimidinic site) lyase	P27695	Quercetin	1.00	Flavonol
	Estradiol 17-beta-dehydrogenase 1	P14061	Apigenin	1.00	Flavone
			Luteolin	1.00	
			Kaempferol	1.00	Flavonol
	Estradiol 17-beta-dehydrogenase 2	P37059	Kaempferol	1.00	Flavonol
		P37059	Quercetin	1.00	Flavonol
	Glyoxalase I	Q04760	Luteolin	1.00	Flavone
			Kaempferol	1.00	Flavonol
			Quercetin	1.00	
	Liver glycogen phosphorylase	P06737	Quercetin	1.00	Flavonol
	Lymphocyte differentiation antigen CD38	P28907	Luteolin	1.00	Flavone
	Myeloperoxidase	P05164	Quercetin	1.00	Flavonol
	NADPH oxidase 4	Q9NPH5	Apigenin	1.00	Flavone
			Luteolin	1.00	
			Kaempferol	1.00	Flavonol
			Quercetin	1.00	
	Phospholipase A2 group B	P04054	Quercetin	1.00	Flavonol
	PI3-kinase p110-gamma subunit	P48736	Quercetin	1.00	Flavonol
	PI3-kinase p85-alpha subunit	P27986	Quercetin	1.00	Flavonol
	Poly [ADP-ribose] polymerase-1	P09874	Luteolin	1.00	Flavone
	Tankyrase-1	O95271	Apigenin	1.00	Flavone
			Luteolin	1.00	
	Tankyrase-2	Q9H2K2	Apigenin	1.00	Flavone
			Luteolin	1.00	
Eraser	Lysine-specific demethylase 4D-like	B2RXH2	Quercetin	0.68	Flavonol
Family A G protein-coupled receptor	Adrenergic receptor alpha-2	P18825	Rutin	1.00	Flavonol
	Alpha-2a adrenergic receptor	P08913	Rutin	1.00	Flavonol
	Neuromedin-U receptor 2	Q9GZQ4	Rutin	1.00	Flavonol

Table A2. *Cont.*

Target Class	Target	Uniprot ID	Metabolite	Probability	Chemical Group
	Adenosine A1 receptor (by homology)	P30542	Luteolin	1.00	Flavone
			Apigenin	1.00	
			Kaempferol	0.80	Flavonol
			Quercetin	1.00	
	Adenosine A2a receptor (by homology)	P29274	Apigenin	1.00	Flavone
			Quercetin	1.00	Flavonol
	Dopamine D4 receptor	P21917	Quercetin	1.00	Flavonol
	G-protein coupled receptor 35	Q9HC97	Quercetin	1.00	Flavonol
	Interleukin-8 receptor A	P25024	Quercetin	1.00	Flavonol
	Vasopressin V2 receptor	P30518	Quercetin	1.00	Flavonol
Hydrolase	Acetylcholinesterase	P22303	Apigenin	1.00	Flavone
			Kaempferol	0.77	Flavonol
			Quercetin	0.68	
			Rutin	1.00	
Isomerase	DNA topoisomerase I (by homology)	P11387	Luteolin	1.00	Flavone
	DNA topoisomerase II alpha	P11388	Quercetin	0.68	Flavonol
Kinase	ALK tyrosine kinase receptor	Q9UM73	Quercetin	1.00	Flavonol
	CaM kinase II beta	Q13554	Quercetin	1.00	Flavonol
	Casein kinase II alpha	P68400	Apigenin	1.00	Flavone
			Quercetin	1.00	Flavonol
	Cyclin-dependent kinase 1	P06493	Quercetin	1.00	Flavonol
	Cyclin-dependent kinase 5/CDK5 activator 1	Q15078	Apigenin	1.00	Flavone
			Luteolin	1.00	Flavone
	Cyclin-dependent kinase 6	Q00534	Apigenin	1.00	Flavone
	Death-associated protein kinase 1	P53355	Quercetin	1.00	Flavonol
	Epidermal growth factor receptor erbB1	P00533	Quercetin	1.00	Flavonol
	Focal adhesion kinase 1	Q05397	Quercetin	1.00	Flavonol
	Glycogen synthase kinase-3 beta	P49841	Apigenin	1.00	Flavone
			Luteolin	1.00	
			Kaempferol	0.66	Flavonol
			Quercetin	1.00	
	Hepatocyte growth factor receptor	P08581	Quercetin	1.00	Flavonol
	Insulin receptor	P06213	Quercetin	0.68	Flavonol
	Insulin-like growth factor I receptor	P08069	Quercetin	1.00	Flavonol
	Myosin light chain kinase, smooth muscle	Q15746	Quercetin	0.68	Flavonol
	NUAK family SNF1-like kinase 1	O60285	Quercetin	1.00	Flavonol
	Protein kinase N1	Q16512	Quercetin	1.00	Flavonol
	Protein kinase C alpha	P17252	Verbascoside	0.78	Phenilpropanoid
	Serine/threonine-protein kinase AKT	P31749	Quercetin	1.00	Flavonol
	Serine/threonine-protein kinase Aurora-B	Q96GD4	Quercetin	1.00	Flavonol
	Serine/threonine-protein kinase NEK2	P51955	Quercetin	1.00	Flavonol
	Serine/threonine-protein kinase NEK6	Q9HC98	Quercetin	1.00	Flavonol
	Serine/threonine-protein kinase PIM1	P11309	Quercetin	1.00	Flavonol
	Serine/threonine-protein kinase PLK1	P53350	Quercetin	1.00	Flavonol

Table A2. *Cont.*

Target Class	Target	Uniprot ID	Metabolite	Probability	Chemical Group
	Tyrosine-protein kinase receptor FLT3	P36888	Apigenin	1.00	Flavone
			Luteolin	1.00	
			Kaempferol	1.00	Flavonol
			Quercetin	1.00	
	Tyrosine-protein kinase receptor UFO	P30530	Quercetin	1.00	Flavonol
	Tyrosine-protein kinase SRC	P12931	Quercetin	1.00	Flavonol
	Tyrosine-protein kinase SYK	P43405	Apigenin	1.00	Flavone
			Luteolin	1.00	
			Kaempferol	0.66	Flavonol
			Quercetin	0.70	
	Vascular endothelial growth factor receptor2	P35968	Quercetin	1.00	Flavonol
Lyase	Carbonic anhydrase I	P00915	Quercetin	1.00	Flavonol
	Carbonic anhydrase II	P00918	Luteolin	1.00	Flavone
				1.00	
			3′-methylquercetin	1.00	Flavonol
			Kaempferol	1.00	
			Quercetin	1.00	
	Carbonic anhydrase III	P07451	Quercetin	1.00	Flavonol
	Carbonic anhydrase IV	P22748	Luteolin	1.00	Flavone
			3′-methylquercetin	1.00	Flavonol
			Kaempferol	1.00	
			Quercetin	1.00	
	Carbonic anhydrase IX	Q16790	Quercetin	1.00	Flavonol
	Carbonic anhydrase VA	P35218	Quercetin	1.00	Flavonol
	Carbonic anhydrase VI	P23280	Quercetin	1.00	Flavonol
	Carbonic anhydrase VII	P43166	Luteolin	1.00	Flavone
			Kaempferol	1.00	Flavonol
			Quercetin	1.00	
	Carbonic anhydrase XII	O43570	Luteolin	1.00	Flavone
			3′-methylquercetin	1.00	Flavonol
			Kaempferol	1.00	
			Quercetin	1.00	
	Carbonic anhydrase XIII (by homology)	Q8N1Q1	Quercetin	1.00	Flavonol
	Carbonic anhydrase XIV	Q9ULX7	Quercetin	1.00	Flavonol
	Carbonic anhydraseVII	P43166	3′-methylquercetin	1.00	Flavonol
Membrane receptor	Beta amyloid A4 protein	P05067	Luteolin	1.00	Flavone
	Receptor-type tyrosine-protein phosphatase F (LAR)	P10586	Ursolic acid	0.70	Triterpene
Nuclear receptor	Estrogen receptor alpha	P03372	Apigenin	1.00	Flavone
	Estrogen receptor beta	Q92731	Apigenin	1.00	Flavone
	Estrogen-related receptor alpha	P11474	Kaempferol	1.00	Flavonol
	Nuclear receptor ROR-gamma	P51449	Ursolic acid	0.70	Triterpene
Other cytosolic protein	Cyclin-dependent kinase 1/cyclin B	Q8WWL	Apigenin	1.00	Flavone
	Cyclin-dependent kinase 1/cyclin B	Q8WWL7	Luteolin	1.00	Flavone

Table A2. *Cont.*

Target Class	Target	Uniprot ID	Metabolite	Probability	Chemical Group
	Heat shock protein HSP 90-alpha	P07900	Catalposide	0.83	Iridoid
			Specioside	1.00	
Other ion channel	Cystic fibrosis transmembrane conductance regulator	P13569	Apigenin	1.00	Flavone
Oxidoreductase	Arachidonate 5-lipoxygenase	P21397	Apigenin	1.00	Flavone
		P35354	Apigenin	1.00	
		P47989	Apigenin	1.00	
		P09917	Luteolin	1.00	
		P21397	Luteolin	1.00	
		P47989	Luteolin	1.00	
		P47989	3′-methylquercetin	1.00	Flavonol
		P09917	Kaempferol	1.00	
		P14679	Kaempferol	1.00	
		P21397	Kaempferol	0.66	
		P47989	Kaempferol	1.00	
		P09917	Quercetin	1.00	
		P21397	Quercetin	1.00	
		P47989	Quercetin	1.00	
Phosphatase	Low molecular weight phosphotyrosine protein phosphatase	P24666	Ursolic acid	0.7	Triterpene
	Protein-tyrosine phosphatase 1B	P18031	Ursolic acid	0.95	Triterpene
	Receptor-type tyrosine-protein phosphatase S	Q13332	Luteolin	0.90	Flavone
	T-cell protein-tyrosine phosphatase	P17706	Ursolic acid	0.74	Triterpene
Primary active transporter	ATP-binding cassette sub-family G member 2	Q9UNQ0	Luteolin	1.00	Flavone
			Kaempferol	1.00	Flavonol
			Quercetin	1.00	
			Apigenin	1.00	Flavone
	Multidrug resistance-associated protein 1	P33527	Apigenin	1.00	Flavone
			Luteolin	0.66	
			Kaempferol	1.00	Flavonol
			Quercetin	1.00	
	P-glycoprotein 1	P08183	Kaempferol	1.00	Flavonol
			Quercetin	1.00	
Protease	Beta-secretase 1	P56817	Quercetin	1.00	Flavonol
	Matrix metalloproteinase 12	P39900	Luteolin	1.00	Flavone
			Poliumoside	0.68	Phenilpropanoid
			Verbascoside	0.74	
	Matrix metalloproteinase 13	P45452	Quercetin	1.00	Flavonol
	Matrix metalloproteinase 2	P08253	Luteolin	1.00	Flavone
			Kaempferol	0.66	Flavonol
			Quercetin	1.00	
			Poliumoside	0.68	Phenilpropanoid
			Verbascoside	0.74	
	Matrix metalloproteinase 3	P08254	Quercetin	1.00	Flavonol

Table A2. *Cont.*

Target Class	Target	Uniprot ID	Metabolite	Probability	Chemical Group
	Matrix metalloproteinase 9	P14780	Luteolin	1.00	Flavone
			Kaempferol	0.66	Flavonol
			Quercetin	1.00	
	Plasminogen	P00747	7-methoxy-luteolin	1.00	Flavone
	Thrombin	P00734	Quercetin	1.00	Flavonol
Secreted protein	Interleukin-2	P60568	6-hydroxyluteolin-7-glucoside	0.70	Flavone
			Apigetrin	0.66	
			Cynaroside	1.00	
			Acacetin-7-O-α-D-glucoside	1.00	O-metilated Flavone
	TNF-alpha	P01375	6-hydroxyluteolin-7-glucoside	0.70	Flavone
			Apigetrin	1.00	
			Cynaroside	1.00	
			Acacetin-7-O-α-D-glucoside	1.00	O-metilated Flavone
	Transthyretin	P02766	Apigenin	1.00	Flavone
			Luteolin	1.00	
Transcription factor	Aryl hydrocarbon receptor	P35869	Kaempferol	1.00	Flavonol
	Zinc finger protein GLI1	P08151	Buddlindeterpene B	0.74	Sesquiterpene
	Zinc finger protein GLI2	P10070	Buddlindeterpene B	0.74	Sesquiterpene
Unclassified protein	Microtubule-assicuated protein tau	P10636	Quercetin	0.68	Flavonol

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Development and Optimization of Supercritical Fluid Extraction Setup Leading to Quantification of 11 Cannabinoids Derived from Medicinal Cannabis

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Simple Summary: This study describes the design and development of setup for the extraction of cannabis strain 1 (Cannabidiol dominant) using supercritical carbon dioxide. For this purpose, two different supercritical fluid extraction instruments were used. The extraction conditions were maintained at 37 °C and 250 bar. Different carbon dioxide inlet and outlet positions were experimented to obtain the maximum yield. A separating chamber was also designed to reduce the throttling effect and dry ice formation during the depressurization process. After developing the supercritical fluid extraction setup, ultra-high performance liquid chromatography coupled with a diode array detection quantification method for 11 cannabinoids was developed.

Abstract: In this study, the optimal setup of supercritical fluid extraction (SFE) was designed and developed, leading to the quantitation of 11 distinct cannabinoids (cannabidivann (CBDV), tetrahydrocannabivann (THCV), cannabidiol (CBD), cannabigerol (CBG) cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabinol (CBN), delta 9-tetrahydrocannabinol (Δ^9-THC), delta 8-tetrahydrocannabinol (Δ^8-THC), cannabichomere (CBC) and delta 9-tetrahydrocannabinol acid (THCA-A)) extracted from the flowers of medicinal cannabis (sp. *Sativa*). Supercritical carbon dioxide ($scCO_2$) extraction was performed at 37 °C, a pressure of 250 bar with the maximum theoretical density of CO_2 (893.7 kg/m^3), which generated the highest yield of cannabinoids from the flower-derived extract. Additionally, a cold separator (separating chamber) was used and positioned immediately after the sample containing chamber to maximize the yield. It was also found that successive washing of the extract with fresh $scCO_2$ further increased yields. Ultra-high performance liquid chromatography coupled with DAD (uHPLC-DAD) was used to develop a method for the quantification of 11 cannabinoids. The C18 stationary phase was used in conjunction with a two solvent system gradient program resulting in the acquisition of the well-resolved chromatogram over a timespan of 32 min. The accuracy and precision of isolated cannabinoids across inter-and intra-day periods were within acceptable limits (<±15%). The assay was also fully validated and deemed sensitive from linearity, LOQ, and LOD perspective. The findings of this body of work are expected to facilitate improved conditions for the optimal extraction of select cannabinoids using $scCO_2$, which holds promise in the development of well-characterized medicinal cannabis formulations. As to our best knowledge, this is the first study to report the uHPLC quantification method for the analysis of 11 cannabinoids from $scCO_2$ extract in a single run with more than 1 min peak separation.

Keywords: cannabis flowers; neutral cannabinoids (sp. *Sativa*); supercritical extraction; supercritical carbon dioxide ($scCO_2$); SFE Nottingham unit; SFE Helix unit

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1. Introduction

Cannabis is considered a highly promising medicinal plant due to its purported array of therapeutic properties, although according to a recent survey it is most commonly used

as an illicit drug [1]. It contains a variety of phytochemicals (around 500 compounds) including sugars, cannabinoids, alkaloids, phenolic compounds, and terpenes [2]. However, the psychoactive and psychotropic property of cannabis is particularly related to the presence of cannabinoids. In medicinal cannabis, cannabidiol (CBD), and tetrahydrocannabinol (THC) are most commonly occurring cannabinoids. From an industrial point of view, CBD is currently considered the most valuable cannabinoid as it possesses a broad range of therapeutic properties, such as anticonvulsant, anxiolytic, neuroprotective, antibiotic, anti-inflammatory activity, and anti-oxidant [3,4]. That said cannabinol (CBN), cannabigerol (CBG), and cannabichromene (CBC) have also shown antifungal, antibacterial, anti-inflammatory, and analgesic properties [5].

The therapeutic properties in natural products are also due to the presence of various phytochemicals, such as glucosinolates, lignans, carotenoids, polyphenols, etc. [6]. Therefore, there is a growing interest to adopt the "natural" or alternative approaches to cure so-called lifestyle diseases, rather than using pharmacologic therapy. The use of natural products for the treatment or to prevent the diseases to gain the healthy lifestyle is progressing [7].

The supercritical fluid extraction (SFE) method has gained increasing interest as a means of extracting cannabinoids from cannabis due to its selective extraction, short processing time, low running cost, and low impact on the environment, compared to conventional solvent-based extraction methods. SFE is a process in which the supercritical fluid (SCF) separates or dissolves components from the plant matrix according to their solvating properties. The solvating property of extracting the component can be maintained by changing the temperature and pressure above the critical point. Therefore, due to the tunable nature of SCF it can only target the desired substance from the sample matrix [8]. Additionally, the design of the SCF extracting chamber also plays an important role in the interaction of SCF substance with a targeted analyte. Proper experimental design can also maximize the yield of the targeted component with high purity. Various theoretical and physical factors (such as inlet and outlet valves of SCF into the extracting chamber, and separating chamber) also participates simultaneously to obtain the high yield. Furthermore, managing the pressure and throttling effect of SCF during extraction collection can enhance the extractability of SCF [9,10].

After the extraction of cannabinoids, the fast and reliable quantification method is an essential step of the analysis. Gas chromatography (GC) is considered as the most useful quantifying and separating technique for the analysis of cannabinoids as it considers a simpler and faster technique compared to high-performance liquid chromatography (HPLC) [11]. However, during GC analysis, acidic cannabinoids convert into their neutral form due to the thermal effect. Therefore, the proper quantification of cannabinoids through GC derivatization step is necessary [12]. HPLC is also considered as the simplest method to analyze the cannabinoids from the cannabis plant and other matrixes, as it does not require high heating step for analysis of the cannabinoids. Therefore, previously a number of HPLC methods have been developed for the determination of cannabinoids [13].

Recent surveys have shown that cannabinoids quantification assays via HPLC focused on the analysis of main cannabinoids (THCA, THC, CBN, CBD, and CBDA) in a single run [14]. However, these methods were either not validated properly or unable to perform the efficient separation of cannabinoids [12,15,16]. Because of the complex nature of the plant extract, the major cannabinoids peaks overlap (such as, CBGA/CBN, and CBG/CBD), which affect the analysis [3].

Previously, a number of studies focused on the SCF conditions for the extraction of cannabinoids. However, the setup of SCF is equally important to gain a high yield of cannabinoids. Therefore, this study was aimed to develop a setup for the SCF extraction of cannabinoids with high yield at optimal operating conditions from cannabis plant material. In addition, reversed-phase uHPLC-DAD quantification assay was developed for the effective quantification of 11 main cannabinoids and their acids with good peak separation.

2. Material and Methods

2.1. Chemical and Reagents

Eleven cannabinoids, namely cannabidivann (CBDV), tetrahydrocannabivarin (THCV), cannabidiol (CBD) with 99.66% purity (Lot: FE08071702), cannabigerol (CBG) with 98.98% purity (Lot: FE06241604), cannabidiolic acid (CBDA) with 98.3% purity (Lot: FE12011601), cannabigerolic acid (CBGA), cannabinol (CBN) with 99.37% purity (Lot: FE06131701), delta 9-tetrahydrocannabinol (Δ9-THC) with 97.66% purity (Lot: FE1041701), delta 8-tetrahydrocannabinol (Δ8-THC), cannabichomene (CBC) with 97.60% purity (Lot: FE10011502), and delta 9-Tetrahydrocannabinol acid (THCA-A) with 99.18% purity (Lot: FE12121601) stock solution with the concentration of 1000 µg/mL in acetonitrile or methanol (acid forms) as reference standards were purchased from Cerilliant, a Sigma Aldrich company (Kinesis Australia Pty Ltd., Redland Bay, QLD, and Novachem Pty Lt, Victoria, Australia). All other solvents (methanol, acetonitrile, and phosphoric acid) were purchased from Merck.

2.2. Sample Collection

Cannabis material has been obtained by the School of Pharmacy, The University of Queensland, under Queensland Health Approval license UNIR008335019; cannabis strain 1 (cannabidiol dominant; had <10% *w/w* of total cannabinoids and among them 90% *w/w* cannabinoids were CBD and CBDA, whereas, THC and THCA were around 5% *w/w*), and strain 2 flower material (had around 14% *w/w* total cannabinoids and among them ~50% *w/w* cannabinoids were cannabidiol and ~45% *w/w* cannabinoids were tetrahydrocannabinol, referred to as the 'balanced strain'). These cannabis samples with Sativa genotype were planted on 4th May, 2017 under the best-growing conditions (12 to 18 h light exposure at 23 °C). Cannabis sample (from plant flowers) collected at the fluorescence stage and dried for 5 to 8 days at 20 °C (with total moisture <10%). This sample was pulverized for 2 min in a coffee grinder (Breville, model BCG200) to obtain a particle size of <2.7 mm. The schematic representation of the cannabis sample preparation and analysis is shown in Figure 1.

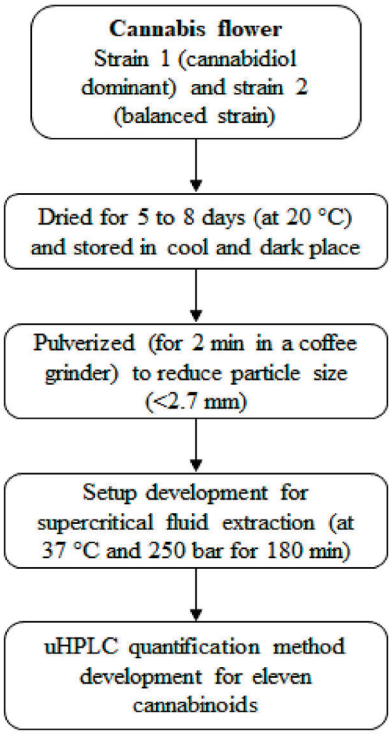


Figure 1. The schematic representation of the cannabis sample preparation and analysis.

2.3. The Supercritical Fluid Extraction (SFE) and Setup Optimization

In this study, the supercritical fluid extraction (SFE) of cannabinoids was performed using $scCO_2$. The density of CO_2 plays an import role for the extraction of selective components from cannabis. Previously, Span and Wagner [15] studied the thermodynamic properties of CO_2 at various states and found that at 250 bar and 37 °C it attained a high density (893.7 kg/m3) in a supercritical state. That is why this study was performed at 250 bar and 37 °C for 3 h to obtain a better extraction of cannabinoids from cannabis. Furthermore, two different SFE operating systems were used for studies to evaluate the optimal set-up for extraction.

2.4. Nottingham Unit

The SFE Nottingham unit (Teledyne ISCO, D-series) was used for the experimental designs A to D. The assembly of Nottingham unit was based on the liquid CO_2 cylinder and syringe compressor to convert into $scCO_2$. However, one main upgrade of the experimental design was the sample holding chamber, and their inlet and outlet positions for CO_2 were designs in the lab. This demonstrates the uniqueness of the designed experimental setup and has led to novel extraction results, which are not reported in literature in the authors' best knowledge. The maximum sample holding capacity of SFE extraction stainless steel vessel for Nottingham unit was 60.0 mL. The syringe pump attached to the liquid CO_2 cylinder can hold up to 250 mL of liquid CO_2 (60 bar) and regulate the desired pressure in extracting chamber. Glass wool and a stainless-steel filter was used on the top of sample holding reactor to separate the extract from grinded plant material.

2.4.1. Experimental Setup A

The 1.0 g sample of cannabis strain 1 was placed inside the extracting chamber and filled with CO_2. The temperature of the extracting chamber was controlled by using a heating jacket to obtain the desired density of CO_2 in sub or supercritical state. The liquid CO_2 dissolved the matrix from the sample according to its density. An overhead stirrer was also used (200 rpm) to help the proper dissolution of a matrix (as represented in Figure 2A). After extraction, the extracting vessel was removed from the SFE extraction system and reverted into collecting vessel to acquire a maximum amount of extracting material with the help of gravity, as shown in Figure 2B.

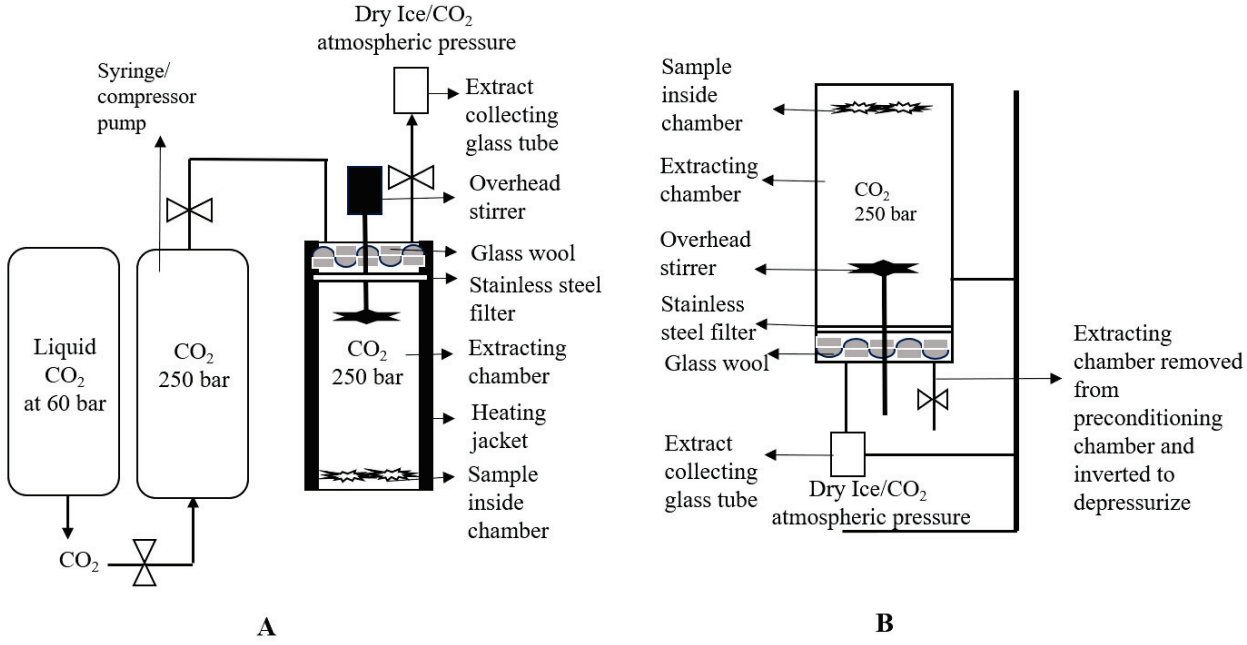


Figure 2. Schematic representation of the SFE Nottingham unit for the experimental setup A (**A**); Sample containing chamber was inverted and placed on stirrer for the depressurization (**B**).

2.4.2. Experimental Setup B

To increase the yield of cannabinoids from the cannabis sample by using SFE, the extraction chamber was rewashed with fresh CO_2. In this procedure, the sample containing chamber was firstly filled with CO_2 (at 250 bar, 37 °C) for 3 h to complete the extraction. After extraction, it was depressurized from 250 bar to 100 bar in the extract collecting chamber, as shown in Figure 2, and then the pressure was maintained again at 250 bar with fresh CO_2 through a pressure regulating syringe. After 30 min, the sample holding chamber was disconnected from a back pressure regulator and again depressurize to 100 bar. After collecting the extract, the sample holding chamber was refilled again with fresh CO_2 at 250 bar to avoid the super-saturation of CO_2 from cannabinoids. This extracting chamber was finally fully depressurized at 0 bar after 30 min of extraction. The safety valve was removed and washed with methanol (5 mL) to collect the extract stuck to it.

2.4.3. Experimental Setup C

In this experimental design, the extraction of cannabinoids was also performed at 250 bar and 37 °C for 3 h. However, the depressurization or washing of the sample after extraction was performed three times. In this process, after 3 h of extraction, the sample holding chamber was removed from the back pressure regulating syringe pump and inverted into a sample collecting chamber with the help of the stand, as presented in Figure 2B. The sample extracting chamber was fully depressurized at 0 bar. After depressurization, the safety valve was removed and washed with 5 mL methanol to obtain the extract if it stuck to the valve. This sample holding chamber was attached again to the pressure regulating syringe pump. The pressure and temperature were maintained again at 250 bar and 37 °C. After 30 min of extraction, depressurization was performed again. The safety valve was also removed and washed with methanol as there was no pressure inside the chamber. After refilling CO_2, full depressurization of the sample holding chamber and washing of the rod with methanol was repeated similarly again to maximize the yield of the cannabinoids.

2.4.4. Experimental Setup D

In this procedure the operating conditions of extraction were similar (250 bar, 37 °C, 3 h) as performed in earlier experiments. However, the glass wool was not used and depressurization of sample holding chamber was performed in an upward direction (as shown in Figure 2A). The full depressurization (at 250 bar to 0 bar) was acquired after 30 min of CO_2 refilling. Overall, rewashing of the sample was performed three times (first after 3 h, second after 30 min and third after 30 min). After complete extraction, the safety valve was opened and washed with 5 mL methanol.

2.5. Helix Unit

The SFE Helix unit (applied separations) was used for the experimental setup B. The maximum sample holding capacity of the helix stainless steel sample holding chamber was 100 mL. The desired internal temperature was monitored by the heating jacket. For the Helix unit, the maximum operating temperature and pressure were 60 °C and 700 bar. The back pressure was directly regulated by the preconditioning chamber from the liquid CO_2 cylinder, as shown in Figure 3.

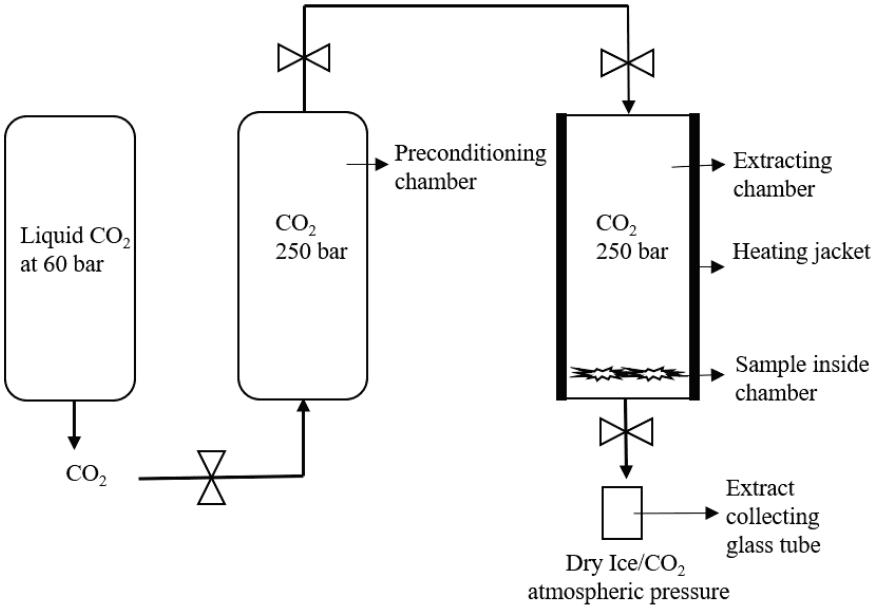


Figure 3. Schematic representation of the Helix unit for setup E extraction.

In this experiment, the SFE of the cannabis sample was performed at 250 bar and 37 °C for 3 h. The 1 gm grinded cannabis flowers sample strain 1 was placed on the bottom of the chamber. The CO_2 stream was entered from the bottom inlet of the chamber and extraction was carried out for 3 h. In addition, there were two built-in filters on the bottom and top end of the sample holding cylinders to separate the plant material and CO_2 from the chamber. After extraction, the CO_2 with the dissolved matrix entered into the separating chamber, where the pressure was around 50 bar to avoid the throttling effect of dry ice (as represented in Figure 3). The extract was collected in the sample collecting vessel, attached to the bottom of the separating chamber. The weight of the extract collecting vessel was measured before and after extraction to estimate the yield of the extract. The sample was washed with the continuous flow of CO_2 for 10 min and a dry sample was collected in the sample collecting vessel.

The SFE Helix unit (applied separations) was used for the experimental setup E to G. Similarly to the assembly of the Nottingham unit, the Helix unit was originally based on a liquid CO_2 cylinder and preconditioning chamber to convert into $scCO_2$. However, the sample holding chamber and their inlet and outlet positions for CO_2 were designs in the lab. The maximum sample holding capacity of the helix stainless steel sample holding chamber was 100 mL. The desired internal temperature was monitored by the heating jacket. For the Helix unit, maximum operating temperature and pressure was 60 °C and 700 bar. The back pressure was directly regulated by the preconditioning chamber from the liquid CO_2 cylinder, as shown in Figure 3.

2.5.1. Experimental Setups E and F

The SCF extraction without using a separating chamber was performed in two different methods. In the first method, the CO_2 stream was entered from the top of the sample holding chamber (as illustrated in Figure 2). However, all the other experimental conditions were similar, as performed in setup E experiment. After 3 h of extraction, the extract was collected from the bottom of the sample extracting chamber in a collection chamber. The weight of extract collecting vessel was measured before and after extraction to estimate the yield of extract.

2.5.2. Experimental Setup G

In this experiment, the SFE of the cannabis sample was performed at 250 bar and 37 °C for 3 h. The 1 gm ground cannabis sample was placed on the bottom of the chamber.

The CO_2 stream was entered from the bottom inlet of chamber and extraction was carried out for 3 h. In addition, there were two built-in filters on the bottom and top end of the sample holding cylinders to separate the plant material and CO_2 from the chamber. After extraction, the CO_2 with the dissolved matrix entered into the separating chamber (as shown in Figure 4), where the pressure was around 50 bar to avoid the throttling effect dry ice. The extract was collected in the sample collecting vessel, attached to the bottom of the separating chamber. The sample was washed with the continuous flow of CO_2 for 10 min and the extract was collected in the sample collecting vessel.

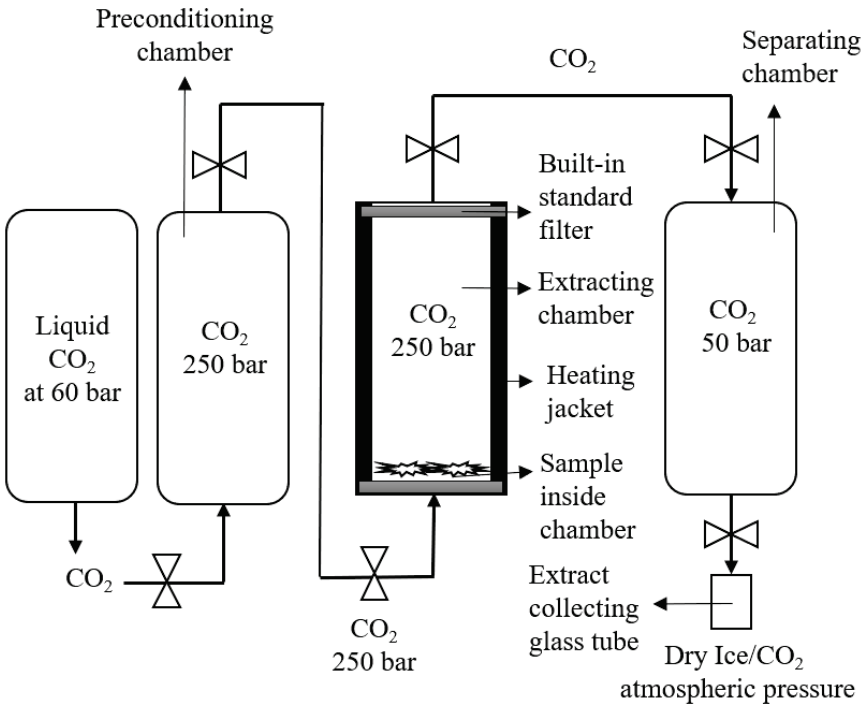


Figure 4. Schematic representation of the Helix unit for setup B extraction.

2.6. uHPLC-DAD Quantification

The uHPLC method was initially developed by Shimadzu Scientific Instruments and transferred to PACE with a loan of a Prominence-I LC-2030 C3D liquid chromatography unit, with a Shim-pack XR ODS-II (2.20 μm, 3.0 mm ID × 75 mm).

2.6.1. Standard Solution Preparation

The concentrated solution of each cannabinoid standard (1000 μg/mL) was diluted in methanol to make 250 μg/mL as a stock solution. The calibration curve of mixed standards with each cannabinoid at 1.0 to 25.0 μg/mL concentrations was prepared in methanol by using the cannabinoids stock solution. All standards solutions were stored at −80 °C.

2.6.2. Instrumentation

Shim-pack XR-ODSII, spherical silica particles, 2.2 μm particle size (Shimadzu Scientific) reversed-phase C18 chromatographic column was used for the separation of cannabinoids. The quantitation method was standardized using Lab Solutions software or Cannabis Analyser (Shimadzu Scientific Instruments, Sydney, NSW, Australia).

2.6.3. Mobile Phase Elution Program

The mobile phase A was the mixture of MilliQ water and phosphoric acid (millimolar; mM) (0.07% H_3PO_4/99.93% MilliQ H_2O; adjusted to pH between 2.22 to 2.26). Mobile phase B was the mixture of methanol and phosphoric acid (mM; 0.07% H_3PO_4/99.92% methanol; adjusted to pH 2.43 to 2.48). The column oven temperature was maintained

at 50 °C and the flow rate was 1.0 mL/min to maintain the column pressure (~5400 to 5600 psi). The volume of injection was 10 µL and the total runtime was 45 min. Initially, mobile phase B (*v/v*) was adjusted at 65% for 1 min. Then, the percentage of mobile phase B was gradually increased from 65% to 72% over the 25 min time period. After that, it finally increases to 95% during a 5 min time period. After maintaining these conditions for 2 min the initial ratio of mobile phases was adjusted and re-equilibrated the column for 12 min.

2.7. uHPLC Method Validation

2.7.1. Selectivity/Identification

To identify the specific cannabinoid in a mixture, the retention time of each standard cannabinoid was ensured separately. For this purpose, the complete UV-visible spectra of each cannabinoid were recorded and compared with the retention time of the mixture.

2.7.2. Precision and Accuracy

The accuracy, repeatability, and intermediate precession of the method were determined by preparing the three samples of each low medium and high concentration (2.5, 10, and 20 µg/mL) of 11 cannabinoids standard mixture on inter and intra days.

2.7.3. Linearity

The linearity of the method was demonstrated by preparing seven different concentrations of standard mixture solution in methanol (containing 11 cannabinoids) from 1.0 µg/mL to 25 µg/mL. The selected range of calibration curves was plotted in triplicates on three consecutive days. The regression of the coefficient (r^2-value) of each calibration curve was calculated to determine the linearity of the method.

2.7.4. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were analyzed by plotting a calibration curve within the range of seven different non-zero detection limit (DL) and quantitation limit (QL) values. The DL and QL of all 11 cannabinoids were calculated by using the following formulas:

$$DL = \frac{3.3\,\sigma}{S}, \tag{1}$$

$$QL = \frac{10\,\sigma}{S}, \tag{2}$$

where σ = response of standard deviation and S = slope of the calibration curve.

2.8. Statistical Analysis

All analyses were performed in triplicates. The statistical analysis was obtained by using Minitab 17 software.

3. Result and Discussion

Due to the complexity and lack of knowledge of SFE factors interactions and in-depth fluid dynamics, SFE is considered a black box design. However, by exploring different experimental parameters, extraction principles and detailed point-to-point process information can produce favorable results [8]. Optimization of the setup to obtain fruitful results is the first stage of every experimental design. Therefore, this study was conducted to design the best setup for the extraction of cannabinoids from cannabis by using SFE. For this study, two different units of SFE were used for the extraction, the Nottingham unit, and the Helix unit. The results are represented in Table 1.

Table 1. Conditions for SCF extraction of cannabinoids from cannabis.

Parameters	Set-Up A	Set-Up B	Set-Up C	Set-Up D	Set-Up E	Set-Up F	Set-Up G
Sample amount (g)	1	1	1	1	1	1	1
Pressure (bar)	250	250	250	250	250	250	250
Temperature (°C)	37	37	37	37	37	37	37
Density of CO_2 (kg/m^3)	893.7	893.7	893.7	893.7	893.7	893.7	893.7
Operating time (min)	180	240	240	240	180	180	180
Stainless steel filter	1	2	2	1	-	-	-
Glass wool amount (gm)	2	1	1	-	-	-	-
Stirring rate (rpm)	200	200	200	200	-	-	-
Number of depressurization	1	3	3	3	1	1	1
Extraction reactor size (mL)	60	60	60	60	100	100	100
Obtained yield (mg)	26.70	33.83 (after first depressurization)	61.82 from CO_2 and 43.80 from rod (after first depressurization)	13.32 from CO_2	-	14.20	53.92
		47.81 (after second depressurization)	69.02 from CO_2 and 20.90 from rod (after second depressurization)	8.73 from CO_2			
		46.03 (after third depressurization)	35.30 from CO_2 and 11.70 from rod (after third depressurization)	9.52 from CO_2 and 7.8 from rod			

3.1. Nottingham Unit

The Nottingham unit was used for experimental setup A and the performed conditions are presented in Table 1. The extraction of cannabinoids from the cannabis sample occurred at 37 °C and 250 bar. These conditions were selected to obtain the maximum density of CO_2. Recent studies only focused on the temperature and pressure of CO_2 for SFE. It was reported that the adoption of very high pressure (up to 500 bar) decreases the selectivity of the cannabis extract [16], because above 250 bar the vapor pressure of the solute also increases. That is why at high pressures, high temperature has a greater influence on the solubility than the density [17]. Therefore, in this study, a carefully low temperature was used to increase the mass transfer rate of cannabinoids from plant to CO_2.In experimental setup A, the glass wool and stainless-steel filter was used for the proper separation of cannabinoids $scCO_2$ extract from original plant material. However, during the depressurization of the $scCO_2$ extract (from 250 bar to atmospheric pressure) it was found that all extracted material (oil) was soaked in glass wool and the yield was quite low (as represented in Figure 5A). To avoid the extract soaking in glass wool and to improve the extraction, experimental setup B was designed.

Figure 5. Schematic representation of glass wool after experimental setup A (**A**) and SFE Nottingham unit extraction (**B**).

In experimental setup B, a comparatively small amount of glass wool and 2 stainless steel filters were used to avoid the contamination of the extract from original ground material. Additionally, the depressurization was performed in triplicates (first; from 250 bar to 100 bar, second; after 30 min extraction with fresh CO_2, from 250 bar to 100 bar, third; again, filled up with fresh CO_2 for 30 min and fully depressurized from 250 bar to 0 bar). As a result, a sharp increase in the total yield of the extract was observed in experimental setup B (127.67 mg) as compared to experimental setup A (26.70 mg). Furthermore, the soaking of extract in glass wool also decreases markedly (Figure 5B).

After the inspiration of experimental set-up B results, experimental setup C was designed, in which full depressurization from 250 bar to 0 bar was performed in triplicates. From the results, it was shown that the total yield and the % of cannabinoids were increased two-fold as compared to experimental set-up B (as shown in Table 1). However, the two main issues were observed. Contamination with original material was not fully resolved and high leakage of cannabinoids on the safety valve and glass wool was found.

To resolve these main issues, the glass wool was fully removed in experimental set-up D and the experiment was performed only using stainless steel filters. However, from the results, it was cleared that the total yield decreased sharply as compared to experimental setups B and C. That is why a further study was performed on Helix unit of SFE as it has built-in filters and contamination chances with original plant material were almost zero or very low.

Helix Unit

After Nottingham, the Helix unit has been used to obtain the high yield of $scCO_2$ extract with a high amount of cannabinoids from cannabis. For this purpose, experimental setups E to G were designed, as shown in Figures 3 and 4. Experimental setup E was very simple because the built-in stainless filter was placed on both ends of the sample holding the chamber. The CO_2 was entered inside the sample holding chamber from the top inlet valve and the sample was placed in a stainless steel cone in the bottom of the sample holding chamber (Figure 6B). However, after the depressurization from the bottom inlet, it was found that the stainless steel filter gets blocked and the plant material stuck on the filter, as illustrated in Figure 6A. Therefore, the extract was not collected.

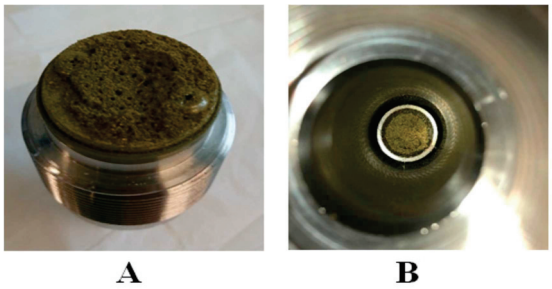


Figure 6. Schematic representation of bottom floor of the sample holding chamber in Helix unit. (**A**): bottom filter was blocked during depressurization of CO_2 from bottom outlet. (**B**): cannabis sample placed at the top of cone.

To figure out this issue, experimental setup F was designed, in which the sample was also placed on the stainless steel cone in the bottom of the sample holding chamber. However, the stream of CO_2 entered from the bottom inlet and depressurization from the top outlet (Figure 4). After the depressurization, it was observed that due to the sudden drop in pressure (250 bar to 0 bar), the throttling process occurred, as presented in Figure 7. As a result, the total yield of $scCO_2$ extract was very low (14.20 mg, Table 1).

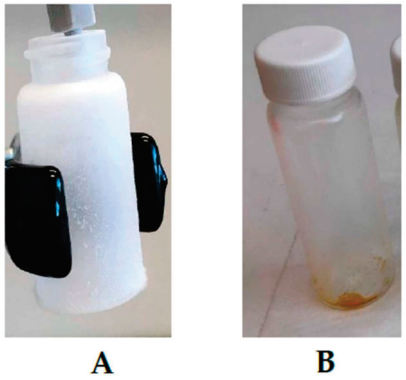


Figure 7. Throttling effect during the extract collection, (**A**): dry ice formation at sudden drop in pressure (250 bar to 0 bar), (**B**): Collection of extract at low pressure drop (50 bar to 0 bar).

Therefore, to improve the setup of SFE extraction with Helix unit, experimental setup F was designed, un which after the sample holding chamber (maximum size 100 mL), the low-pressure regulating chamber/separating chamber (maximum holding pressure 100 bar) was adjusted. As a result, the good yield (53.92 mg, Table 1) was obtained after single depressurization.

3.2. Ultra-High-Performance Liquid Chromatography Coupled with DAD (uHPLC-DAD)

uHPLC with DAD is considered as the most accurate and simple method for the quantification of cannabinoids. Because it is easy to perform on a routine basis, it efficiently separates the analyte and does not degrade the sample during quantification [18]. Several studies developed methods for the quantification of cannabinoids. Such as, De Backer, Benjamin [19] designed and validated a method to analyze the eight cannabinoids by using three chemotypes (including, fiber-type, intermediate-type, and drug-type) extracts (chloroform: methanol; 1:9 *v/v*) of the cannabis plant. Additionally, this method was validated with a clearly separated HPLC profile. In another study Ciolino, Ranieri [20] developed a new HPLC-DAD quantitation method to determine the 11 cannabinoids in cannabis samples by using two different columns. The analytical column, ACE 5 C18-AR (250 mm × 4.6 mm ID, 5 µm) gives better separation than the conventional c-18 column. The isocratic mobile phase system for ACE 5 C18-AR and Luna C-18 was 34: 66 and

26:74 for 0.5% acetic acid: acetonitrile. The total run time was 50 min and the figures of chromatogram are shown in Figure 8.

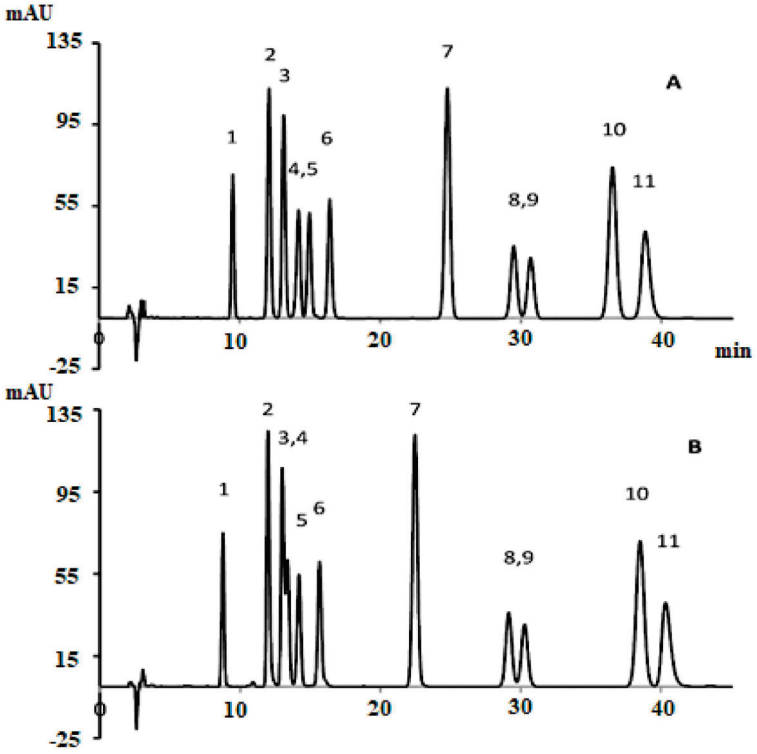


Figure 8. HPLC profile of 11 cannabinoids using an analytical column, (**A**) ACE 5 C18-AR (250 mm × 4.6 mm ID, 5 µm) and (**B**) conventional c-18 column [20]. Elution order: 1-CBDV, 2-CBDA, 3-CBGA, 4-CBG, 5-CBD, 6-THCV, 7-CBN, 8-d9THC, 10-CBC, and 11-THCA. Reprinted with permission from Ref. [20]. Copyright 2021 Copyright Ciolino.

However, only five cannabinoid compounds were validated (CBN, THCA, CBDA, d9THCA, and CBD). These cannabinoids were also scrutinized in cannabis oils, extracts, plant, and their commercial products. In different states (free-flowing liquids or viscous compounds, semisolids, solids, emulsions, dispersions, aqueous and non-aqueous solutions) and polarities such as polar foodstuffs (beverages and sugary foods) nonpolar products (butter, balms/certain ointments), and substances with intermediate polarities (oral supplements and many topical foods).

HPLC analysis of 11 cannabinoids cannabis extract and biomass was also performed by Gul, Gul [21]. The mobile phase system was gradient (water and acetonitrile with 0.1% formic acid). The separation chromatogram was obtained by using Luna C-18 column at 220 nm in 22.2 min run time. The elution order is also represented in Figure 9. This is quite similar to Figure 8 but overall, the efficiency of the separated peaks was low. Whereas, this method was validated for all selected cannabinoids and their concentration was also measured in 13 various samples of cannabis.

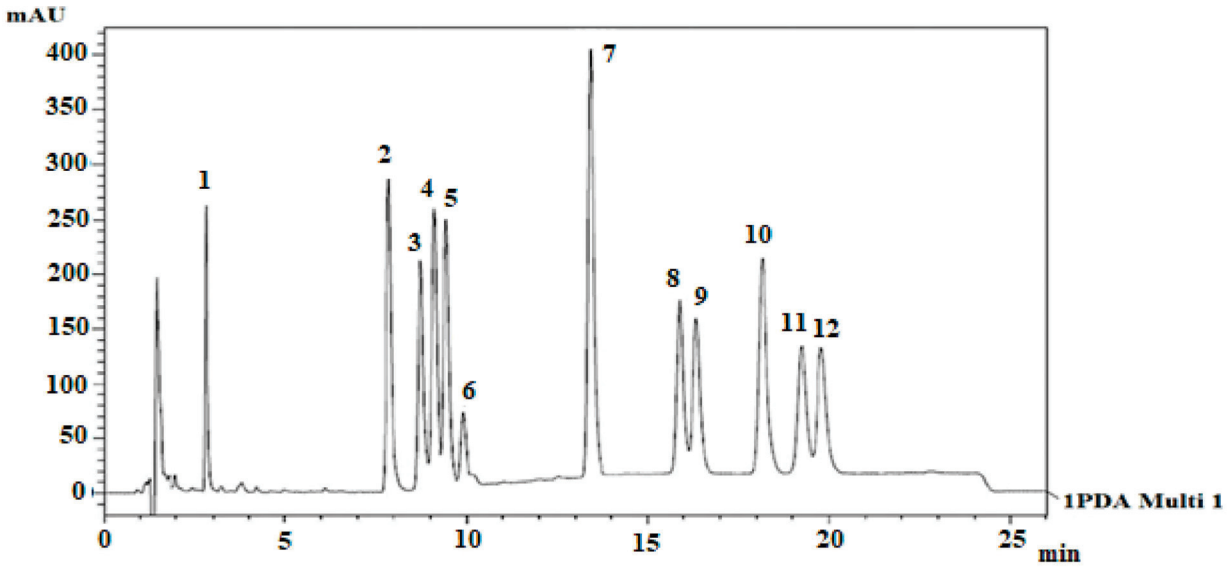


Figure 9. HPLC profile of 11 cannabinoids by using Luna C-18 analytical column [21]. Elution order: 1-I.S, 2-CBDA, 3-CBGA, 4-CBG, 5-CBD, 6-THCV, 7-CBN, 8-d9THC, 9-d8THC, 10-CBL, 11-CBC, and 12-THCA.Reprinted with permission from Ref. [21]. Copyright 2021 Copyright Gul.

McPartland, MacDonald [22] used reversed-phase HPLC to investigate the binding affinity of THC and its acidic precursor THCA-A with CB_1 and CB_2 receptors in humans. In this method, the C18 column was used with a linear gradient mobile phase system and the chromatogram was obtained within 25 min. However, this study only focused on the stability of non-psychoactive cannabinoids (THCA-A) and their binding capability in human body receptors. However, the study revealed a greater binding affinity of THC CB_1 (62-fold) and CB_2 (125-fold) as compared THCA-A.

Various other studies developed methods to determine six to seven main cannabinoids and their acidic precursors by using HPLC. However, these methods are not validated. Such as Romano and Hazekamp [23] had been used preheated cannabis (with 19% THC) extracts in olive oil, olive oil with water, ethanol, petroleum ether naphtha for the quantification of cannabinoids through HPLC.

Therefore, this study aimed to develop a new method for the quantification of 11 main cannabinoids in cannabis and its derived products with good peak separation. In this study, psychoactive and non- psychoactive neutral cannabinoids and their acidic form were separated in 32 min (Figure 10).

3.3. Method Validation

3.3.1. Selectivity/Identification

The peaks of all 11 cannabinoids were fully separated during 32 min of program run. The retention time of each cannabinoid is shown in Table 2 and presented in Figure 10. To identify each peak of cannabinoid in a standard mixture, all cannabinoids were analyzed separately. Their elution order, retention time, and sensitivity were also confirmed through system suitability. It was also noticed that the pH of both mobile phases plays a very important role in stable separation and in maintaining a good retention time.

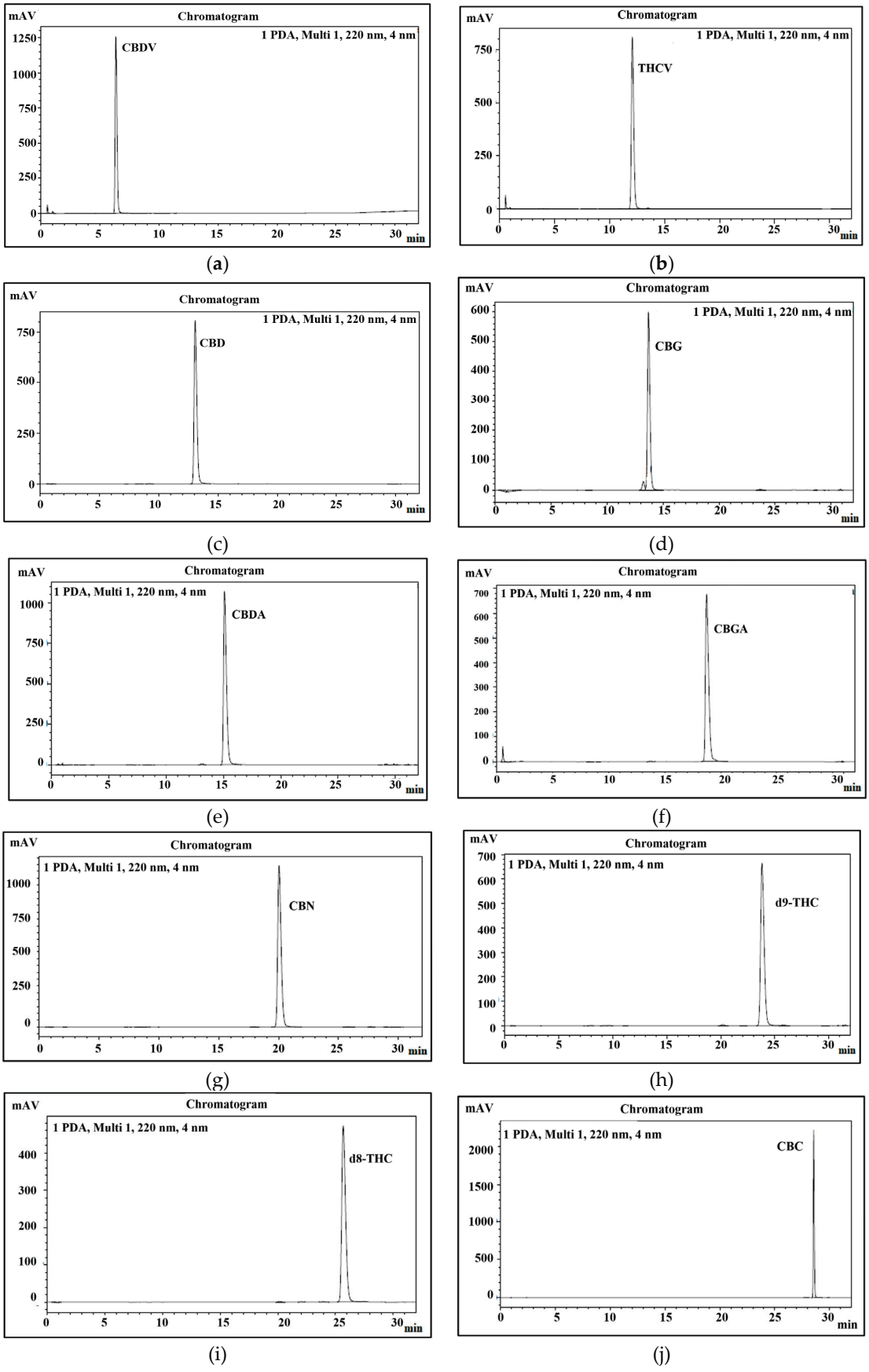
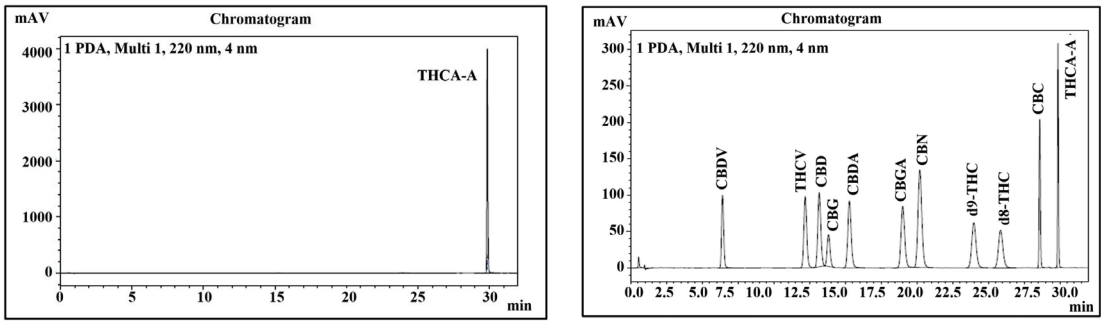


Figure 10. *Cont.*



(k) (l)

Figure 10. Peaks identification of eleven cannabinoids. (**a**) uHPLC profile of CBDV; (**b**) uHPLC profile of THCV; (**c**) uHPLC profile of CBD; (**d**) uHPLC profile of CBG; (**e**) uHPLC profile of CBDA; (**f**) uHPLC profile of CBGA; (**g**) uHPLC profile of CBN; (**h**) uHPLC profile of Δ^9-THC; (**i**) uHPLC profile of Δ^8-THC; (**j**) uHPLC profile of CBC; (**k**) uHPLC profile of THCA-A; (**l**) uHPLC profile of 11 cannabinoid mixture.

Table 2. System suitability for higher concentration (20 μg/mL) of 11 cannabinoid standard mixtures.

Name of Cannabinoid	System Suitability (Peak Area ± SEM)	Retention Time (min) ± SEM
CBDV	656,922 ± 465	5.79 ± 0.018
THCV	899,571± 764	11.18 ± 0.036
CBD	963,188 ± 641	12.10 ± 0.045
CBG	400,828 ± 347	12.76 ± 0.050
CBDA	994,274 ± 804	14.15 ± 0.052
CBGA	996,755 ± 546	17.79 ± 0.071
CBN	1,641,927 ± 1305	18.87 ± 0.074
Δ^9-THC	845,242 ± 948	22.56 ± 0.088
Δ^8-THC	747,971 ± 711	24.41 ± 0.090
CBC	848,289 ± 708	28.25 ± 0.029
THCA-A	959,980 ± 1004	29.75 ± 0.017

3.3.2. Precision and Accuracy

The precision and accuracy of intra and inter days are represented in Table 3. The method for each cannabinoid was validated at three different levels of concentration, including low (2.5 μg/mL), medium (10 μg/mL), and high (20 μg/mL) as shown in Table 3. The %RSD of all selected 11 cannabinoids for the intra-day varied from 1.60% to 3.37% and for the inter-day from 0.20% to 1.75% respectively. Similarly, the variations in the accuracy level of each cannabinoid were also in an acceptable range. For the intra-day, the accuracy level for the low limit varied from 91.2 to 103.0 μg/mL, for the medium limit from 101.9 to 103.0 μg/mL, and the higher limit from 97.7 to 104.1 μg/mL. Whereas, for the inter-day, the accuracy level for the low limit varied from 88.26 to 99.6 μg/mL, for the medium limit from 101.12 to 103.04 μg/mL, and for the higher limit from 96.8 to 106.0 μg/mL. They are in the acceptable limit of 85.0 to 115.0% (±15%), except for LOQ 80.0 to 120.0% (±20%).

3.3.3. Linearity, the Limit of Detection (LOD), and Limit of Quantitation (LOQ)

The sensitivity of the method was obtained by determining the linearity, LOD, and LOQ (results are represented in Table 4). The obtained LOD of this analytical method ranged between 0.27 to 0.51 μg/mL, showing that a very low quantity of cannabinoids in extract can be measured by this method, without any guarantee of the imprecision or bias in the result of this assay.

Table 3. Precision and accuracy of 11 cannabinoids on intra-day and inter-day.

Name of Cannabinoid	Intra-Day				Inter-Day			
	Concentration (μg/mL)	Average Calculated Concentration (μg/mL)	Accuracy (%)	Precision (%) RSD	Concentration (μg/mL)	Average Calculated Concentration (μg/mL)	Accuracy (%)	Precision (%) RSD
CBDV	2.5	2.25	90.3	3.06	2.5	2.20	88.3	0.52
	10	10.29	102.9	2.04	10	10.19	101.9	0.66
	20	19.88	99.4	2.13	20	19.72	98.6	0.72
THCV	2.5	2.27	91.1	3.14	2.5	2.22	89.1	0.51
	10	10.29	103.0	2.03	10	10.18	101.8	0.72
	20	20.74	104.0	2.15	20	20.58	102.9	0.73
CBD	2.5	2.27	91.0	3.26	2.5	2.22	89.1	0.68
	10	10.18	101.9	2.47	10	10.11	101.1	0.64
	20	20.07	100.4	2.14	20	19.90	99.5	0.74
CBG	2.5	2.39	95.7	3.37	2.5	2.34	93.9	0.24
	10	10.29	103.0	2.45	10	10.21	102.1	0.65
	20	19.54	97.7	2.22	20	19.57	97.8	0.35
CBDA	2.5	2.43	97.3	2.27	2.5	2.39	95.7	0.63
	10	10.26	102.7	2.43	10	10.17	101.7	0.58
	20	19.87	99.4	1.86	20	19.73	98.7	0.67
CBGA	2.5	2.53	101.4	2.56	2.5	2.49	99.6	1.75
	10	10.23	102.4	1.90	10	10.18	101.8	0.57
	20	20.81	104.1	1.81	20	20.67	103.4	0.63
CBN	2.5	2.46	98.7	3.38	2.5	2.40	96.1	0.24
	10	10.30	103.	2.69	10	10.30	103.0	0.74
	20	20.96	104.8	2.14	20	21.20	106.0	0.71
Δ^9-THC	2.5	2.37	95.1	3.90	2.5	2.31	92.4	0.43
	10	10.24	102.5	2.06	10	10.12	101.2	0.56
	20	20.82	104.1	2.36	20	20.63	103.2	0.81
Δ^8-THC	2.5	2.52	100.7	3.15	2.5	2.46	98.5	0.20
	10	10.24	102.5	2.06	10	10.13	101.4	0.66
	20	20.76	103.8	2.20	20	20.93	104.7	0.72
CBC	2.5	2.48	99.4	2.93	2.5	2.42	96.9	1.56
	10	10.22	102.2	2.01	10	10.13	101.4	0.65
	20	19.48	97.4	1.94	20	19.35	96.8	0.63
THCA-A	2.5	2.43	97.3	3.21	2.5	2.37	95.1	0.64
	10	10.26	102.6	1.60	10	10.22	102.2	0.65
	20	20.08	100.4	1.83	20	19.97	99.9	0.48

A calibration curve of 11 cannabinoid standard mixtures was performed to evaluate the concentration of cannabinoids in unknown samples or ground plant material. The calibration curve was conducted in triplicates on three consecutive days, with the stable, linear, and r^2-value always >0.99 for each standard. Additionally, from the results of LOD (0.27 to 0.51 μg/mL) and LOQ (0.92 to 1.71 μg/mL), it was shown that the method was sensitive. The obtained LOD and LOQ values of cannabinoids were also comparable with previously developed methods [3,21].

Table 4. Linearity, the limit of detection (LOD), and limit of quantitation (LOQ).

Name of Cannabinoid	Linearity (r^2)	LOD (µg/mL)	LOQ (µg/mL)
CBDV	0.992 ± 0.001	0.42	1.41
THCV	0.992 ± 0.001	0.33	1.11
CBD	0.995 ± 0.000	0.34	1.13
CBG	0.996 ± 0.001	0.31	1.03
CBDA	0.997 ± 0.000	0.32	1.08
CBGA	0.994 ± 0.001	0.32	1.06
CBN	0.993 ± 0.000	0.33	1.102
Δ^9-THC	0.992 ± 0.001	0.37	1.26
Δ^8-THC	0.995 ± 0.001	0.51	1.71
CBC	0.995 ± 0.001	0.29	0.99
THCA-A	0.993 ± 0.000	0.27	0.92

Where, Δ^9-THC is *Delta-9-tetrahydrocannabinol* and Δ^8-THC is *Delta-8-tetrahydrocannabinol*.

3.4. Analysis of Cannabinoids

Two different cannabis strains were used for the SCF extraction (as shown in Table 5) and their chromatogram is also represented in Figure 11, in which strain 1 was CBD + CBDA dominant (around 90% *w/w*) and strain 2 had an almost equal amount of CBD + CBDA (50% *w/w*) and THC+THCA (45% *w/w*) as compared to other cannabinoids. The results of well cannabis strains from cannabis strain extracts obtained from the final SFE setup are also presented in the table.

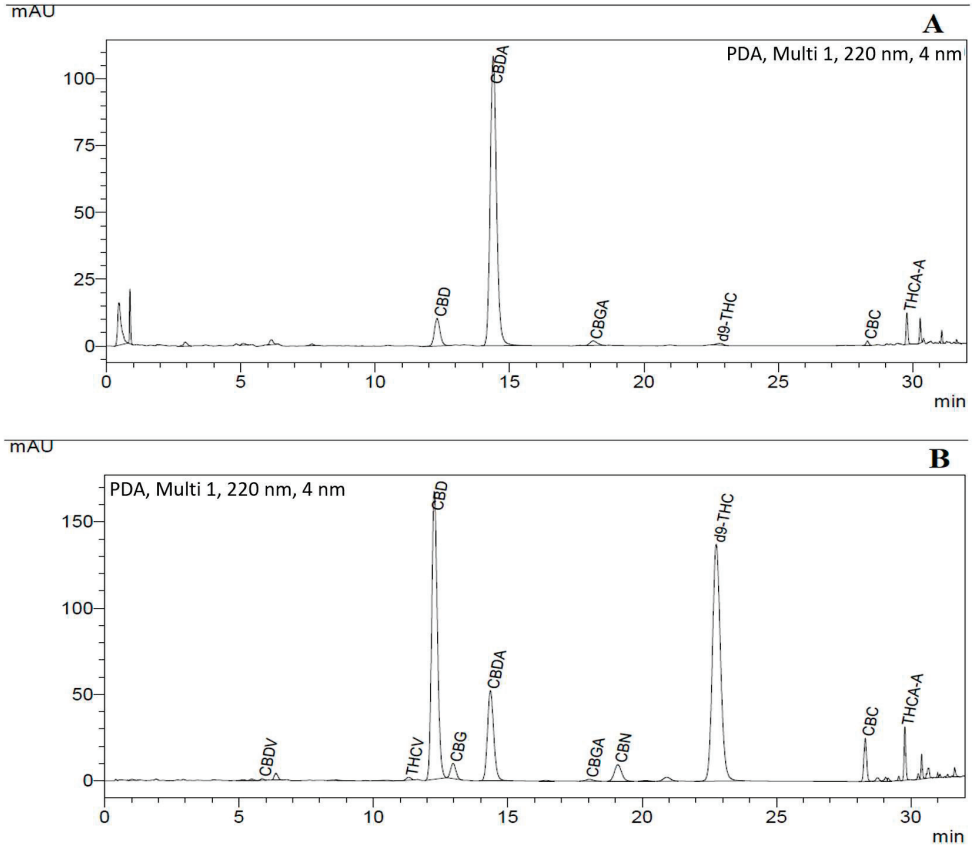


Figure 11. uHPLC profile of cannabis strains: (**A**) with dominant CBD + CBDA (around 90% *w/w*); and (**B**) with 55% *w/w* CBD+CBDA and 35% THC + THCA in the cannabinoid mixture.

Table 5. Amount of cannabinoids in the SCF extract of two different cannabis strains.

Cannabinoids	CBD µg/mL (±S.E.M)	% CBD (*w*/*w*)	CBDA µg/mL (±S.E.M)	% CBDA (*w*/*w*)	THC µg/mL (±S.E.M)	% THC (*w*/*w*)	THCA µg/mL (±S.E.M)	% THCA (*w*/*w*)
Strain 1	92.23 ± 0.02	0.369	282.50 ± 0.13	1.130	9.48 ± 0.00	0.038	4.71 ± 0.01	0.019
Strain 2	252.72 ± 0.39	1.011	21.05 ± 0.25	0.084	197.50 ± 0.11	0.790	1.60 ± 0.00	0.006

4. Conclusions

The optimal setup configuration of supercritical fluid extraction (SFE) was achieved by systematically changing the inlet and outlet valve position of SFE for CO_2 entrance and depressurization. However, $scCO_2$ extraction conditions were fixed at 250 bar, 37 °C, 180 min, and 1 g plant material, to measure the cannabinoids yield during setup optimization. Additionally, the purity of the extract was also increased by using stainless steel built-in filters and an additional separating chamber. Furthermore, for the quantification of neutral cannabinoids and their acids, a highly sensitive reverse-phase uHPLC-UV-DAD method was developed. All selected cannabinoids showed good separation over the 32 min runtime (45 min with re-equilibration). Their relative retention time was also strongly influenced on the pH of mobile phases and the operating pressure of the column. The method was validated by analyzing the linearity, LOQ, LOD, accuracy, and precision in triplicates on inter and intra-day according to US-FDA guidelines.

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Article

Jasonia glutinosa (L.) DC., a Traditional Herbal Tea, Exerts Antioxidant and Neuroprotective Properties in Different *In Vitro* and *In Vivo* Systems

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Simple Summary: *Jasonia glutinosa* (L.) DC or rock tea (RT) is a plant traditionally used to treat different pathologies. In this study the neuroprotective potential of an ethanolic extract of RT is analyzed. Caenorhabditis elegans model and *in vitro* assays with relevant central nervous system enzymes were used. The results showed antioxidant and neuroprotective potential of this plant.

Abstract: In traditional medicine, *Jasonia glutinosa* (L.) DC or rock tea (RT) has been mainly used to treat digestive and respiratory pathologies but also as an antimicrobial or an antidepressant herbal remedy. An ethanolic extract of RT has been demonstrated to have antioxidant and anti-inflammatory effects, which may be explained by its phytochemical profile, rich in polyphenols and pigments. The aim of this study is to investigate the neuroprotective potential of RT. For this purpose, the ethanolic extract of RT is assayed in *Caenorhabditis elegans (C. elegans)* as an *in vivo* model, and through *in vitro* assays using monoamine oxidase A, tyrosinase and acetylcholinesterase as enzymes. The RT extract reduces juglone-induced oxidative stress in worms and increases the lifespan and prevents paralysis of *C. elegans* CL4176, a model of Alzheimer's disease; the extract is also able to inhibit enzymes such as acetylcholinesterase, monoamine oxidase A and tyrosinase *in vitro*. Together these results demonstrate that *Jasonia glutinosa* is a good candidate with antioxidant and neuroprotective potential for the development of new products with pharmaceutical interests.

Keywords: medicinal plants; *C. elegans*; acetylcholinesterase; monoamine oxidase A; tyrosinase; herbal medicine; lifespan

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1. Introduction

Jasonia glutinosa (L.) DC. (Compositae), whose popular name is "té de roca" (rock tea, RT), is a medicinal plant distributed in Mediterranean countries, mainly Spain and France [1]. Ethnobotanical studies on this plant report that its main use, as an infusion, is for the treatment of diarrhea, dyspepsia or abdominal pain [2]. These digestive properties were demonstrated for the first time in a murine model of colitis [3,4]. In these studies, the oral administration of an ethanolic extract of RT (5, 25 and 50 mg/kg) ameliorated colitis symptomatology, prevented the macroscopic damage and histological changes induced by dextran sulfate sodium in mice and normalized the intestinal contractibility and the intestinal total transit disrupted by the colitis [3,4].

In addition to its use as a digestive, *J. glutinosa* has been widely used for the treatment of respiratory or infective pathologies, hypertension, pain, emesis or even mood disorders [2,5–7]. Different studies reported an ethnopharmacological use of RT as a "stimulant", capable of improving mood and clearing the mind [6,7].

Some of these effects could be explained by anti-inflammatory [4,8] or antioxidant effects [4,5,9], which are related to its phytochemical composition. A recent *in vivo* study has shown that dietary supplementation with RT (10 or 30%) for 15 or 30 days in sea bream (*Sparus aurata L.*) improves short-term immunostimulatory capacity (15 days) and maintains antioxidant capacity in the long-term (30 days) [10].

Different studies have shown that *J. glutinosa* is rich in polyphenols, terpenes, esters, alkanes, lactones or flavonol glucopyranoside [1,4,9]. High-performance liquid chromatography with a diode-array detector (HPCL-DAD) analysis of the ethanolic extract of RT showed a rich content of phenolic compounds (134.4 mg/g, dry extract) and pigments (0.27 mg/g, dry extract) (Figure 1). Among the 15 phenolic compounds detected, 10 were phenolic acids and 5 flavones. 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 1,5-di-O-caffeoylquinic acid were the phenolic acids most represented (70% of the total phenolic content). The most abundant flavonoid is quercetin-3-O-galactoside (50% of total flavonoid). In respect to pigments, two carotenoids, chlorophylls and xanthophylls were detected. Lutein represented 55% of the total pigments [4].

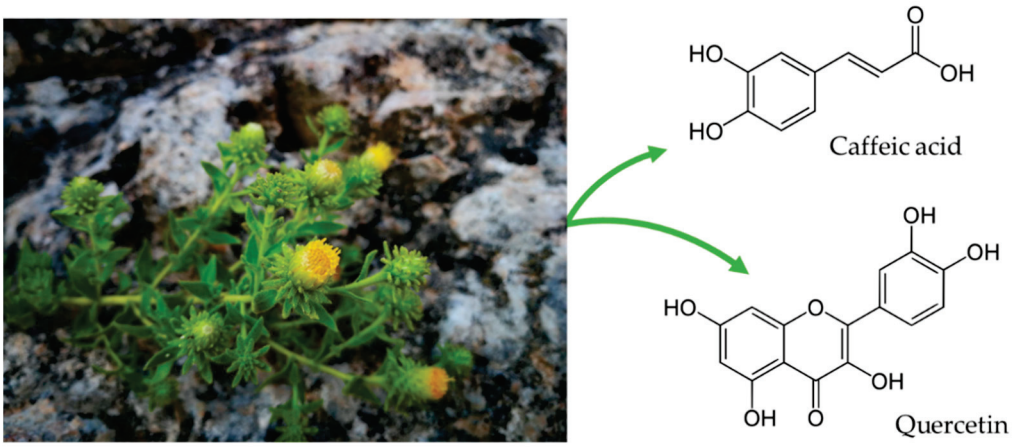

Figure 1. *J. glutinosa* photography taken by authors and the main phenolic compounds of the extract.

Oxidative stress and inflammation have been linked to the aging process as triggers of various diseases, including neurodegeneration. *J. glutinosa*, with a phytochemical composition rich in polyphenols, has previously demonstrated antioxidant and anti-inflammatory effects, and its use as an antidepressant has also been reported by certain inhabitants of Spain. However, there are no scientific reports showing the potential of RT as a neurotherapeutic agent. Therefore, the objective of this work is to study the antioxidant and neuroprotective activity of RT extract in a *Caenorhabditis elegans (C. elegans)* model, studying the effect of *J. glutinosa* on stress resistance, lifespan and amyloid toxicity and *in vitro* analyzing its ability to inhibit the enzymes of the nervous system such as acetylcholinesterase (AChE), monoamine oxidase A (MAO-A) and tyrosinase (TYR), involved in neurotransmitters' metabolism. These enzymes participate in the elimination of biogenic amines such as acetylcholine, serotonin and catecholamines (dopamine, epinephrine, norepinephrine), which are involved in the development of different pathologies of the nervous system such as Alzheimer's, Huntington's and Parkinson's. Therefore, the inhibitory substances of these enzymes could help the treatment of these diseases.

2. Materials and Methods

2.1. Reagents and Chemicals

The following chemical reagents: gallic acid, xanthine, NBT (nitroblue tetrazolium), xanthine oxidase, DPPH (2,2 Diphenyl 1 picrylhydrazyl), galantamine, ATCI (acetylthiocholine iodide), DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)), Tris, vanillic acid, 4-aminoantipyrine, horseradish peroxidase, acetylcholinesterase, tyramine, MAO-A, L-DOPA (levodopa) and tyrosinase were obtained through Sigma-Aldrich (Madrid, Spain). Clorgyline and α-Kojic acid were sourced from Cymit quimica (Barcelona, Spain). Na_2CO_3, HCl, NaCl, Methanol and potassium phosphate were acquired from Panreac (Barcelona, Spain). Juglone (5-hydroxy-1,4-naphthoquinone) and FUdR (5-fluoro-2′-deoxyuridine) were sourced from Alfa Aesar (Ward Hill, MA, USA). *C. elegans* strains and Escherichia coli OP50 were obtained from the Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA).

2.2. Plant Material and Extraction

Jasonia glutinosa ethanolic extract was obtained as described in Valero et al., (2015) [11] and a plant voucher was kept in the Universidad San Jorge (ref. 001-2012). The ethanolic extract was analyzed phytochemically in a previous author's study using HPLC-DAD, determining its composition in phenolic compounds and pigments [4].

2.3. Caenorhabditis elegans Studies

2.3.1. *C. elegans* Strains and Maintenance Conditions

This study used the wild-type strain of *C. elegans* (N2) and a transgenic strain (CL4176 (smg-1 ts 131 (myo-3/Aβ1–42 long 3′-UTR)). The *C. elegans* CL4176 strain contains a temperature-sensitive transgene that expresses the human amyloid peptide β1–42 in muscle, which causes paralysis in worms.

The strains were maintained at 16 °C (CL4176) or at 20 °C (N2) on nematode growth media (NGM) agar plates seeded with *Escherichia coli* OP50 as a food source. Synchronized worms were obtained using an alkali-bleaching method [12] for the N2 strain and egg-laying for the CL4176 strains.

2.3.2. Assessment of Resistance to Lethal Oxidative Stress

Synchronized L1 worms were cultivated in NGM agar plates in the presence of different concentrations of RT extract (5, 10, 20 and 50 μg/mL) or in its absence (control). Under these conditions, the worms were incubated at 20 °C until the first day of adulthood. RT-treated and control adult worms were washed with sterile water and transferred to 96-well microplate with NGM agar containing 150 μM juglone (5-hydroxy-1,4-naphthoquinone), which produces lethal oxidative stress. After an incubation period of 24 h at 20 °C, survival was evaluated by the response to a mechanical stimulus [13]. The number of alive and dead worms was recorded and the survival rate % (% SR) was calculated:

$$\% \text{ SR} = (\text{N}^{\text{o}} \text{ of worms alive x } 100)/\text{Total number of worms} \quad (1)$$

Each concentration was tested in triplicate and 100 nematodes were used per assay.

2.3.3. Lifespan Analysis

The lifespan of the wild-type *C. elegans* (N2) was tested using different concentrations of RT (5, 10, 20, 50 and 100 μg/mL) and compared with untreated controls following the method of Solis and Petrascheck [14]. Synchronized wild-type L1 larvae were transferred to 96-well plates (7–18 worms/well) and were cultured in an S-complete medium containing *E. coli* OP50 (1.2×10^9 bacteria/mL). On the first day of adulthood, FUdR (0.06 mM) was added to sterilize the adults (day 0). RT extracts were added 24 h later. Survival of nematodes was scored every two or three days. The scoring method was the same as used for the juglone oxidative stressed assays. Results are expressed as survival rate % (Equation (1))

and mean lifespan. Each assay was repeated three times and used 150 nematodes per condition.

2.3.4. Paralysis Assay

In vivo neuroprotective effects of RT extract were evaluated using the strain CL4176 according to previous research protocols [15]. The strain CL4176 was egg-synchronized onto the NGM plates seeded with *E. coli* containing 0, 5, 10 and 25 μg/mL of the RT extract and cultured at 16 °C. The temperature was changed to 25 °C to induce expression of the amyloid-β (Aβ) transgene 38 h later (day 0). Paralysis was scored twice a day for five days [16]. Worms that exhibited pharyngeal pumping, but did not move, or only moved the head after being touched with a platinum wire, were scored as paralyzed. The test was performed with 100 nematodes per condition.

2.4. Bioassays Regarding CNS Enzymes

2.4.1. Acetylcholinesterase Inhibition

Ellman's method was performed in 96-well microplates using a microplate reader to measure absorbance as previously described [17]. Each assay well contained a mixture of 25 μL 15 mM ATCI in Milipore water, 125 μL 3 mM DTNB in buffer C (50 mM Tris-HCl, pH = 8, 0.1 M NaCl, 0.02 M $MgCl_2$ $6H_2O$), 50 μL buffer B (50 mM Tris-HCl, pH = 8, 0.1% bovine serum) and 25 μL RT extract at different concentrations (20.00, 10.00, 5.00, 2.50, 1.25 and 0.63 μg/mL) in buffer A (50 mM Tris-HCl, pH = 8). At last, 25 μL AChE enzyme (0.22 U/L) was added to begin the reaction. Controls and blanks were performed, containing buffer A instead of samples and a buffer instead of the enzyme, respectively. Absorbance was read 13 times every 13 s at 405 nm. Galantamine was used as a reference inhibitor.

2.4.2. Monoamine Oxidase A Inhibition

The MAO-A activity was performed in a 96-well microplate using a technique previously described [18]. The assay mixture contained 50 μL RT extract at different concentrations (0.0001, 0.0010, 0.0100, 0.1000, 1.0000 and 10.0000 mg/mL), 50 μL chromogenic solution (0.8 mM vanillic acid, 417 mM 4-aminoantipyrine and 4 U/mL horseradish peroxidase in potassium phosphate buffer pH = 7.6.), 100 μL 3 mM tyramine and 50 μL 8 U/mL MAO-A. Controls and blanks were also performed, with solvent instead of samples and a buffer instead of the enzyme, respectively. The absorbance was read at 490 nm every 5 min for 30 min. Clorgyline was used as a reference inhibitor.

2.4.3. Tyrosinase Inhibition

The TYR activity was assessed in 96-well microplates using a procedure previously described [19]. The reaction mixture contained 10 μL RT extract at different concentrations (0.001, 0.010, 0.100, 0.500, 1.000, 2.500 and 5.000 mg/mL), 40 μL of L-DOPA, 80 μL phosphate buffer, pH = 6.8 and 40 μL tyrosinase; these were mixed in each well. Controls and blanks were also performed with 50 μL solvent instead of samples and 50 μL buffer instead of the enzyme, respectively. Absorbance was read at 475 nm. α-Kojic acid was used as a reference inhibitor.

2.5. Statistical Analysis

Data were expressed as mean ± SEM and for statistical analysis, GraphPad Prism version 6.0c (GraphPad Software, San Diego, CA, USA) was used. Extract concentration was required to inhibit 50% of the activity of the nervous system enzymes, IC_{50}; this was estimated using non-linear regression. ANOVA following by Tukey's multiple comparisons test were used to evaluate resistance to oxidative stress. Lifespan and paralysis curves were tested using log-rank for significant fit to Kaplan–Meier survival curves. The significance level was set to $p < 0.05$.

3. Results

3.1. Rock Tea Extract Improved the Stress Resistance of C. elegans

To evaluate the antioxidant effect of *J. glutinosa*, wild-type *C. elegans* were pre-treated with RT extract (5, 10, 20 and 50 μg/mL) for 24 h and then exposed to a lethal dose of juglone, a natural pro-oxidant. As Figure 2 shows, this treatment resulted in 0.92% ± 0.52 survival of the control group. Pre-treatment with RT extract improved the survival rate of the worms in a dose-dependent manner. Groups pre-treated with the highest doses of RT extract, 20 and 50 μg/mL, significantly increased the survival rate compared to the control group, with rates of 9.15% ± 2.13 ($p < 0.001$) and 10.69% ± 2.51 ($p < 0.0001$), respectively. These results indicate that the RT extract improves oxidative stress resistance in *C. elegans*, protecting them from oxidative stress.

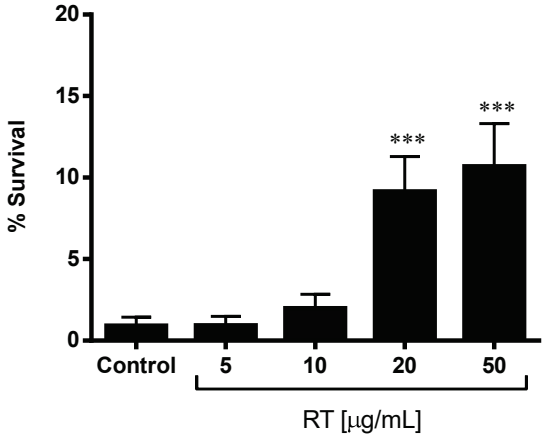

Figure 2. Rock tea (RT) extract increases the survival of wild-type *C. elegans* exposed to lethal oxidative stress. L1 worms were incubated on treatment plates with different doses of RT (5, 10, 20 and 50 μg/mL) until they reached adulthood and then exposed to juglone (150 μM) for 24 h. The results represent the mean ± SEM of the values from three independent experiments. The significance for the differences between the control and pre-treated worms is *** $p < 0.001$.

3.2. Rock Tea Extract Increased C. elegans Lifespan

With the object to study the effect of RT extract on lifespan, wild-type *C. elegans* grown at 20 °C in a liquid medium containing different doses of RT (5, 10, 20, 50 and 100 μg/mL) were used. *C. elegans* is a model widely used to study aging and age-related disorders due to the good conservation of the biochemical pathways and their short life cycle [20]. The lifespan curves showed that RT extract increases the lifespan of the worms in a dose-dependent manner (Figure 3). Significant differences were found ($p < 0.05$) in survival curves between the control group and the worms treated with 100 μg/mL of RT extract (12.8 ± 0.56 days vs. 14.7 ± 0.58 days, respectively). The maximum lifespan, understood as the average lifespan of 10% of each population living longer, increased by 11.36% at doses of 20 and 50 μg/mL of RT and 13.81% at the dose of 100 μg/mL of RT with respect to the control. These results show that RT extract presents a positive effect on *C. elegans* lifespan.

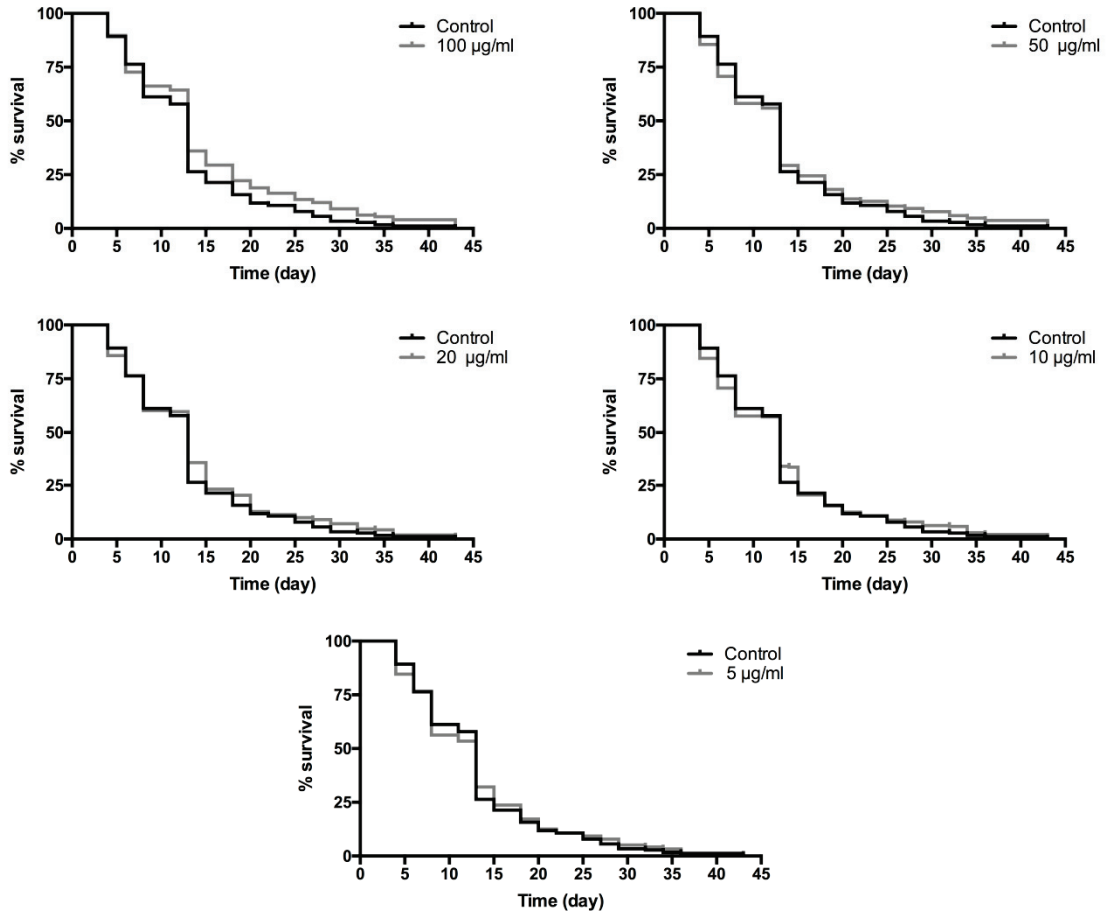

Figure 3. Lifespan curves of wild-type *Caenorhabditis elegans (C. elegans)* in a liquid medium supplemented with different concentrations of RT extract (0–100 µg/mL) at 20 °C. Synchronized worms were exposed to the extract from the second day of adulthood (day one). Scoring of survival was carried out three times per week until all worms died. The results are representative of three independent biological replicates. The curves were analyzed using a long-rank test. Differences in the survival curves between the treatment and control groups were found at the dose of 100 µg/mL, with a *p*-value of 0.0153.

The means of lifespan were 12.8 ± 0.56 days in the control and 13 ± 0.57, 13 ± 0.56, 13.45 ± 0.58, 13.42 ± 0.57 and 14.7 ± 0.55 days in the RT 5, 10, 20, 50 and 100 µg/mL groups, respectively. The results of lifespan experiments were analyzed using the Kaplan-Meier survival model and for significance by means of a log-rank pairwise comparison test between the control and treatment groups. Differences in survival curves between the treatment and control groups were found for 100 µg/mL with a *p*-value of 0.0153.

3.3. Rock Tea Extract Delays the Onset of Paralysis Induced by Aβ Peptide

Although there are multiple factors involved in Alzheimer's disease, numerous studies have shown that neuronal accumulation of the Aβ peptide plays a central role in the development of the disease [21]. To further investigate the *in vivo* neuroprotective effect of rock tea, an examination was carried out as to whether diet supplementation may affect the progression of paralysis induced by Aβ toxicity in the *C. elegans* transgenic strain CL4176. This strain has proven to be a good model for screening potential neuroprotective natural products [15,22,23]. Nematodes were exposed to different concentrations of RT extract (5, 10 and 25 µg/mL) from the egg stage. Then, human Aβ expression was induced by a temperature upshift that makes worms paralyze over time. The time to develop paralysis was analyzed using survival curves (Figure 4).

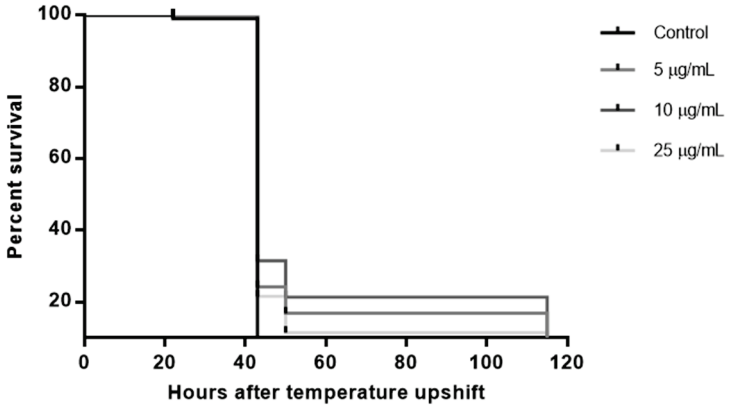

Figure 4. Effect of RT extracts on Aβ-induced paralysis in transgenic *C. elegans* CL4176. The statistical significance of the difference between the curves was analyzed using a log-rank (Kaplan–Meier) statistical test, which compares the survival distributions between the control and treatment groups. Differences in the survival curves between the treatment and control groups were found ($p < 0.0001$).

The time for 50% nematodes to become paralyzed (PT_{50}) showed a significant increase ($p < 0.0001$) in the groups treated with RT extract compared to the untreated groups. However, between the different groups treated with RT extract, no significant differences were found in PT_{50} at 43 h. According to HR (hazard ratio) values obtained by log-rank analysis (0.76; 0.72 and 0.78), the RT extract significantly reduced the risk of paralysis by 23%, 28% and 21% in worms treated with 5, 10 and 25 μg/mL RT extract concentration, respectively.

3.4. Bioassays Regarding CNS Enzymes

The AChE, MAO-A and TYR enzymes are involved in processes that regulate neurotransmission in the CNS. RT extract was able to inhibit all the enzymes at high doses, although the dose-response curve was shifted to the right with respect to the reference substances for each enzyme (Figure 5). AChE inhibition of RT extract is achieved at high doses, with an IC_{50} of 4.5 mg/mL for RT (Figure 5B). The galantamine IC_{50} for AChE inhibition was 0.1 mg/mL. RT extract also revealed the potential to achieve a total inhibition of MAO-A (Figure 5B). RT extract IC_{50} was 76.34 μg/mL, a little far from that of the reference inhibitor, clorgyline, which was 0.12 μg/mL. Finally, RT extract also showed the inhibitory potential of TYR in a dose-dependent manner (Figure 5C). The extract reached 100% enzyme inhibition at a high dose, 5 mg/mL, and the IC_{50} of the assay were 1.05 and 0.004 mg/mL for RT extract and kojic acid, respectively.

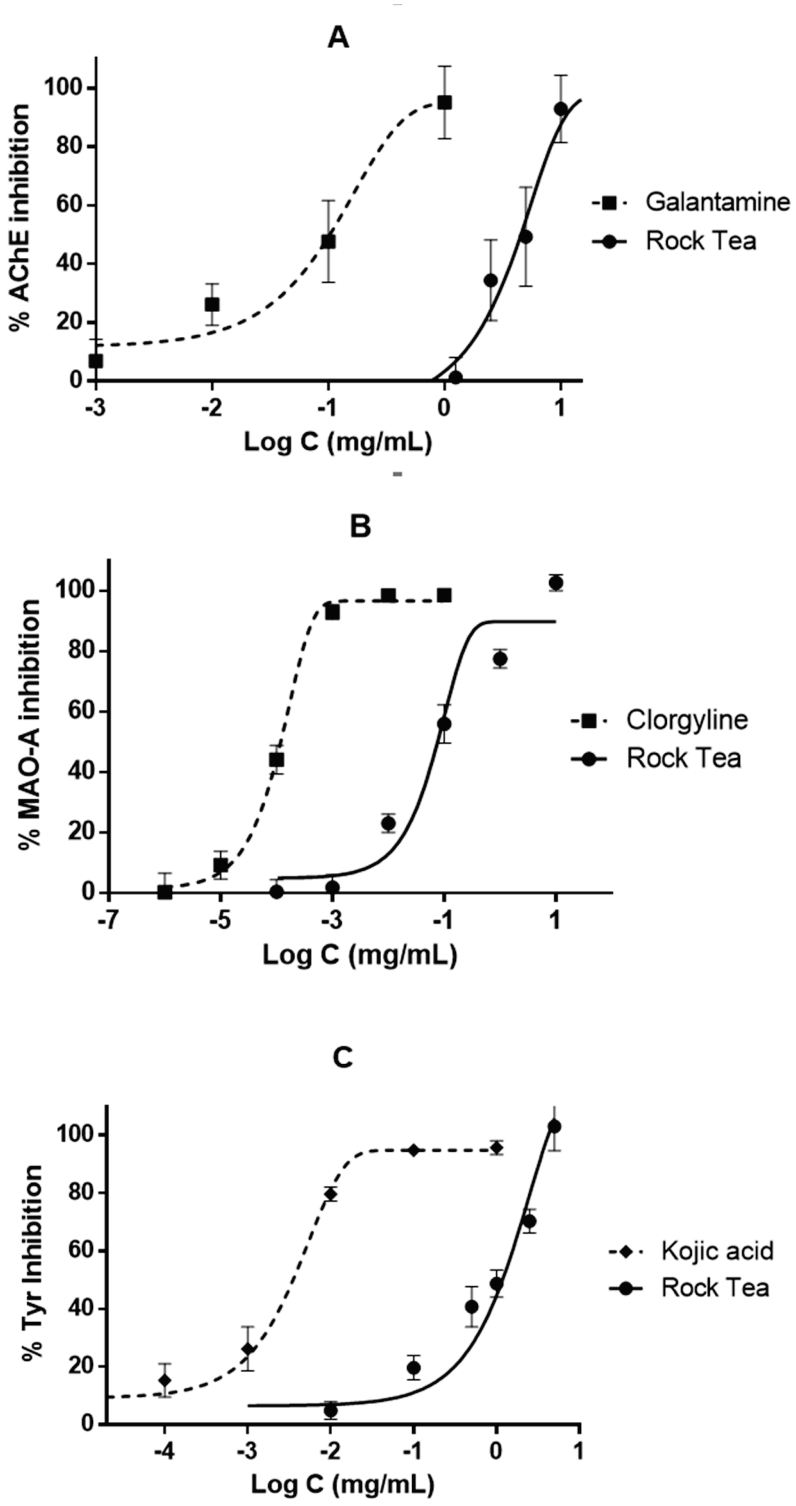

Figure 5. Neuroprotective effect of RT extract on nervous system enzymes; (**A**) Acetylcholinesterase (AChE) inhibition, (**B**) Monoamine oxidase A (MAO-A) and (**C**) Tyrosinase (TYR) inhibition. Galantamine, clorgyline and kojic acid have been used as reference inhibitors, respectively.

4. Discussion

This study shows, for the first time, the neuroprotective effect of *Jasonia glutinosa* extract using different types of *in vitro* and *in vivo* bioassays. The results obtained demonstrate that RT extract prevented oxidative stress, increased the lifespan and delayed paralysis of

the transgenic amyloid *C. elegans*. In addition, the extract inhibited enzymes of the nervous system such as acetylcholinesterase, monoamine oxidase A and tyrosinase.

Various studies have associated reactive oxygen species with the pathogenesis of Alzheimer's disease (AD) by showing that reactive oxygen species promote the formation and accumulation of the β-amyloid peptide and hyperphosphorylation of Tau. On the other hand, oxidative stress is associated with aging. Many theories try to explain the connection, but it is complicated due to its multifactorial etiology. The so-called "Oxi-Inflamm-Aging" theory explains the events that occur during aging and the appearance of diseases related to it; during cellular aging, senescent cells produce pro-inflammatory cytokines that produce chronic systemic inflammation ("inflamm-aging"), leading to an increase in free radicals. Similarly, oxidative stress produces states of inflammation due to the impaired immune system, creating a vicious circle between oxidative stress, inflammation and aging. This process has been implicated in multiple disease states, such as cardiovascular disease, cancer, neurodegenerative diseases, diabetes or respiratory diseases [24,25]. Oxidative stress, β-amyloid peptide accumulation the lifespan have been correlated in the *C. elegans* model [26,27], making it an excellent model to study the bioactive properties of substances with neuroprotective potential.

Different RT extracts have shown anti-inflammatory effects [4,10] and a high capacity to eliminate superoxide and DPPH radicals [4,5,9]. Furthermore, one *in vivo* study showed these antioxidant properties by decreasing HSP70 levels, increasing peroxidase activity and upregulating the Nrf-2 transcription factor, producing an increase in the expression of antioxidant enzymes such as catalase and superoxide dismutase in fish [10]. These properties, which could be explained by its rich composition in phenolic acids, flavonoids and pigments, make RT an ideal candidate as a neuroprotective natural agent.

Thus, the RT extract has demonstrated antioxidant properties and lifespan extension in *C. elegans* in a similar manner to quercetin or kaempferol, two flavonoids present in the extract. The phytochemical analysis of the ethanolic extract showed a composition rich in phenolic compounds and pigments [4]. Of the phenolic compounds identified, the most representative were the hydroxycinnamic acids derived from caffeoylquinic acid, represented mainly by 3,4-di-O, 3,5-di-O (40.95 mg/g of dry extract), 1,5-di-O (24.73 mg/g of dry extract) and 4,5-di-O caffeoylquinic acids (23.14 mg/g of dry extract). Of the flavonoids, the most representative compound was quercetin-3-O-galactoside (15.16 mg/g of dry extract). In addition, pigments in the extract were determined for the first time, highlighting the presence of carotenoids and chlorophylls, lutein being the most representative, with content greater than 55% of the total.

The treatment of *C. elegans* with quercetin or its methyl derivates, isorhamnetin and tamarixetin (200 μM), increased worm survival rate under exposure to juglone (150 μM, 24 h exposure). Quercetin showed a greater capacity than methyl derivatives in decreasing oxidative proteins compared to untreated worms. Isorhamnetine showed greater protection (16%) compared to quercetin and tamarixetine (11%), prolonging the mean lifespan of the worms compared to the control worms [13]. These results were similar to those obtained by Kampkötter and colleagues (2008), who demonstrated that quercetin (100 μM) enhanced the percentage of survival against oxidative stress (juglone 150 μM, 72 h) by 19% and the mean lifespan by 15% of worms compared to the untreated worms [28]. Furthermore, worms pre-treated with kaempferol showed diminished oxidative stress and less accumulation of reactive oxygen species [29]. Kaempferol and quercetin showed scavenging capacity in mitochondrial reactive oxygen species levels, without affecting lifespan in *mev-1* mutant worms, a mutation that increases sensitivity to oxidative stress and reduces lifespan [30]. The effect on lifespan may be explained by the antioxidant effect of these compounds and/or by other complementary mechanisms, independent of antioxidant properties, such as stress-sensitive signaling pathways.

One of the main key regulatory pathways in *C. elegans* lifespan and the response to oxidative stress is the insulin/insulin-like growth factor (IGF-1) signaling (IIS) pathway, as well as the activation of different transcription factors such as DAF-16/FoxO, HSF-

1 and SKN-1/Nrf-2 that regulate stress-related genes as antioxidant enzymes. Several studies have shown that quercetin and kaempferol produced a translocation of DAF-16 from the cytosol to the nucleus in transgenic *C. elegans*, increasing the expression of defense proteins [28–30], the role of these DAF-16 target genes in longevity is unclear. Different studies have shown that transcription of this factor may not be responsible for the effect of flavonoids on worm lifespan, since quercetin-treated *daf-16* deletion mutants increased survival in comparison with the control [30–32]. It has recently been shown that resistance to oxidative stress by quercetin in worms would implicate genes involved in the IIS pathway such as *age-1*, *akt-1*, *akt-2*, *daf-18*, *sgk-1*, *daf-2* and *skn-1*, independent of transcription factors such as DAF-16 and HSF-1.

Other important compounds in RT extract are caffeoylquinic acids (CQAs), which are emerging as an interesting group of bioactive compounds with high potential as neuroprotective agents. These compounds have been shown to possess strong antioxidant and neuroprotective properties *in vitro* and *in vivo*. CQAs have also been shown to improve cognitive impairment in several rodent models of AD [33]. Recently, Chen et al. [34] showed that 1,5-O-dicaffeoyl-3-O-(4-malic acid methylester)–quinic acid (MQA), a derivative of caffeoylquinic acid, can protect against ischemic brain injury in rats. This neuroprotective effect may involve the inhibition of cell apoptosis due to p38 activation, the increase of the Bcl-2/Bax ratio and the modulation of NFk-B1 and caspase-3 expression. Furthermore, the observed decrease in lipid peroxidation suggests that the antioxidant activity of MQA could also contribute to the protective effect against cerebral ischemia. The association between antioxidant effects and neuroprotection has been previously demonstrated for other CQAs. Thus 3-O-caffeoylquinic acid and caffeic acid treatment improved memory in the mouse model of Aβ accumulation by the inhibition of oxidative stress, inflammation and apoptosis through the p38 mitogen-activated protein kinase (MAPK) signaling pathway [35,36]. Similarly, Chu et al. [37] observed that a flavone-rich extract of *Tetrastigma hemsleyanum* whose major components were 3-caffeoylquinic acid, 5-caffeoylquinic acid and quercetin-3-O-rutinoside, and kaempferol-3-O-rutinoside could attenuate glutamate-induced toxicity in PC12 cells and *C. elegans*. This extract lessened genotoxicity, relieved oxidative stress and recovered mitochondrial functions in PC12 cells via the MAPK pathway by suppressing the over-phosphorylation of ERK and p38. Furthermore, treatment suppressed O_2^- generation, reduced GSH depletion and partially restored the normal motility lost after the glutamic acid exposition in *C. elegans*. Therefore, antioxidant properties could explain the positive effect of *J. glutinosa* extract in increasing the lifespan in N2 and transgenic *C. elegans* (CL4176 strain).

As mentioned above, oxidative stress and the malfunction of specific enzymes related to neurotransmitter effects, as AChE, are involved in the development of the pathogenesis of AD. The accumulation of Aβ peptide caused by free radicals would result in neuron dysfunction and death. Different works have shown that phenolic compounds present neuroprotective activity, decreasing the deleterious effects of oxidative stress, increasing ACh availability, reducing the anti-inflammatory effect and interacting directly with Aβ peptides inhibit their aggregation and oligomerization [15,23,38,39]. The reduction of the IIS pathway and expression of genes as DAF-16, HSF-1, HSP-16 and Nrf2/SKN-1 have also been related to a neuroprotective effect by decreasing the expression of Aβ peptides and improved the paralysis and lifespan in a *C. elegans* model of AD [40]. These results show that RT extract decreases the risk of paralysis in transgenic strain CL4176, and its neuroprotective effect can be explained by the synergic antioxidant and neuroprotective activities of phenolic acids and flavonoids.

On the other hand, *C. elegans* present numerous neurotransmitters, some of which are implicated in different pathologies of the nervous system such as ACh, dopamine, GABA or glutamate [41]. RT extract has demonstrated the ability to inhibit the AChE, MAO and TYR enzymes, which could explain its traditional uses as a stimulant, memory enhancer or antidepressant, reinforcing its neuroprotective role. Others isolated extracts of plants and flavonoids have demonstrated the capacity to inhibit CNS enzymes [15,18,23,42,43]

and decrease the toxicity by Aβ accumulation [15,23,38,44]. Due to these mechanisms, many natural products have neurotherapeutic potential for the prevention and treatment of different CNS disorders.

5. Conclusions

Jasonia glutinosa is a medicinal plant with a wide range of traditional uses. The neurotherapeutic potential of a polyphenolic extract has here been demonstrated in different models due to its protective activity against oxidative stress and the capacity to inhibit β-amyloid aggregation in *C. elegans*. This herbal extract has also shown the inhibitory capacity of nervous system enzymes, which might explain, from an *in vitro* perspective, possible mechanisms of its use as a stimulant and antidepressant. All in all, this plant species as well as its extracts and bioactive compounds are worthy of further investigation for the prevention of diseases associated with cellular aging and oxidative stress.

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List of Abbreviations : AChE: acetylcholinesterase, AD: Alzheimer's disease, Aβ: amyloid-β, CQAs: caffeoylquinic acids, NGM: nematode growth media, MAO-A: monoamine oxidase A, MQA: 1,5-O-dicaffeoyl-3-O-(4-malic acid methylester)–quinic acid, RT: rock tea, TYR: tyrosinase, % SR: survival rate %.

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biology

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Review

Effects of Propolis on Infectious Diseases of Medical Relevance

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Simple Summary: Propolis is a beekeeping product with a complex and highly variable chemical composition. Many beneficial health properties have been reported. In this review, we will be focusing on compiling the studies carried out with propolis on infectious diseases of greater medical relevance. Likewise, the promises and challenges that propolis has to consolidate itself as a complementary therapy for the treatment of these diseases are analyzed.

Abstract: Infectious diseases are a significant problem affecting the public health and economic stability of societies all over the world. Treatment is available for most of these diseases; however, many pathogens have developed resistance to drugs, necessitating the development of new therapies with chemical agents, which can have serious side effects and high toxicity. In addition, the severity and aggressiveness of emerging and re-emerging diseases, such as pandemics caused by viral agents, have led to the priority of investigating new therapies to complement the treatment of different infectious diseases. Alternative and complementary medicine is widely used throughout the world due to its low cost and easy access and has been shown to provide a wide repertoire of options for the treatment of various conditions. In this work, we address the relevance of the effects of propolis on the causal pathogens of the main infectious diseases with medical relevance; the existing compiled information shows that propolis has effects on Gram-positive and Gram-negative bacteria, fungi, protozoan parasites and helminths, and viruses; however, challenges remain, such as the assessment of their effects in clinical studies for adequate and safe use.

Keywords: propolis; antibacterial; antifungal; antiparasitic; antiviral; bioactive compounds

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1. Introduction

Currently, most health systems around the world are based mainly on the prevention of diseases. The world is constantly exposed to a large number of pathogens that cause emerging and re-emerging disease. These pathogens differ widely in terms of severity and probability and have varying consequences for morbidity and mortality, jeopardizing not only health but also social and economic well-being. It is absolutely necessary to have a global health system that is able to prevent and respond effectively to the expanding and evolving infectious diseases, as well as solving an increasingly widespread antimicrobial resistance [1]. The need to prevent, identify, and respond to any infectious disease that compromises global health stability remains a national, regional, and international priority [2].

Existing natural products could be potential resources to find different compounds for the development of new drugs and relevant medicine [3], creating an area of study of great importance, since the immense difference of natural molecules could contribute bioactive compounds that help in therapeutic improvement [4]. Propolis is a natural resinous product elaborated by bees from material obtained from various botanical sources; it is mixed with bees' wax and enzymes secreted by the bee's salivary glands [5]. Characteristically, its composition is 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% other substances [6]. The propolis was informed to present about 300 distinct compounds [7]. The characteristic chemical groups identified in propolis are phenolic acids or their esters, flavonoids, terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes, and β-steroids [7,8]. In addition, both the biomedical effect and composition of propolis have a very high variability according to the region of collection, the surrounding plant sources, and the seasons [9,10]. Many reports have shown that propolis possesses antibacterial, antifungal, antiparasitic, antiviral, antioxidant, anti-inflammatory, antitumor, antidiabetic, and immunomodulatory properties [11–19]. Propolis is a bee product that contains a great variety of biomedical properties and a great spectrum of components that could be promising candidates for drug discovery, which could be used to treat characteristic affections of distinct diseases. Notably, infectious diseases are a public health problem, since they do not have adequate treatment because many pathogens have developed resistance to the different drugs used against them. This is where propolis and many other alternative and complementary medicine products play an important role, since they are easily accessible, allowing a high percentage of the world population to use them, providing options to complement current treatments. As such, it is necessary to clinically analyze the effectiveness of propolis to evaluate its potential in human health promotion.

2. Antibacterial Activity of Propolis

One of the main complications with diseases caused by bacteria is their resistance to the antibiotics commonly used against them. Antibiotics are chemical compounds that can act in two ways: inhibiting (bacteriostatic drugs) or killing (bactericidal drugs) bacteria. These drugs are characterized by a specific interaction with a defined target in the bacterial cell, and they are arguably the most important medical intervention introduced by humans [20]. Currently, the figures related to this problem are alarming: according to conservative numbers mentioned by the Centers for Disease Control (CDC), approximately 23,000 people are estimated to die annually only in the USA as a result of an infection with an antibiotic-resistant organism [21]. According to a report, antibiotic resistance is predicted to cause around 300 million premature deaths by 2050, with a loss of up to USD 100 trillion to the global economy [22]. Next, we address the main research describing the use and activities of propolis from different countries on some bacterial agents of greater medical relevance today.

2.1. Staphylococcus Infections

The genus *Staphylococcus* causes different infections in the human population like impetigo, scalded skin syndrome, toxic shock syndrome, pneumonia, endocarditis, and urinary tract infections, among others [23]. Some species of this genus are resistant to antibiotics, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [24]. In this genus, we can highlight to *S. aureus* and *Staphylococcus epidermidis*.

Records exist that demonstrate the use of propolis since ancient civilizations, as it possesses a large number of biological properties, one of which is its antibacterial effect [9,25–27]. Currently, various investigations around the world have demonstrated the antibacterial capacity of different types of propolis; hence, various studies report that all the distinct varieties of propolis have different antibacterial activities [28]. On the American continent, propolis varies widely, each having different characteristics. In this context, the antibacterial activity of Canadian propolis was evaluated, which showed activity on *S. aureus* [29]. Likewise, the antibacterial effect of Brazilian propolis (red, green, and brown)

from distinct areas was studied, with the authors finding that the red extracts demonstrated activity against different bacterial species, including *S. aureus*; however, the green and brown extracts showed less activity than red extracts [30]. Similarly, in Europe, French propolis demonstrated significant antibacterial activity against both methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA [31]. Likewise, Polish propolis showed variability in its activity on twelve MSSA and MRSA clinical isolates [32].

In 2017, Al-Ani et al. mentioned the antibacterial activity of propolis of various geographic origins such as Germany, Ireland, and the Czech Republic. The three propolis samples showed moderate antibacterial effect on *S. aureus*, MRSA, and *S. epidermidis* [28]. Similarly, Italian propolis showed antibacterial activity on clinically isolated *S. aureus* and *S. epidermidis*. The propolis demonstrated an inhibition on lipase activity of 18 *Staphylococcus* spp. and an inhibition on the coagulase of 11 tested *S. aureus*. Propolis showed an inhibitory activity of the adhesion and consequent biofilm growth of *S. aureus* [33]. In another study, 53 propolis were obtained from various areas in Serbia, which revealed one type of blue propolis and one orange, depending on floral and geographical origin. Propolis samples showed an effect against different bacteria, including *S. aureus*, with the orange-type propolis samples showing higher antibacterial activity compared with the blue-type propolis samples (Table 1) [34]. The variety of climates and flora in Africa results in propolis with very particular characteristics; however, as with samples from the Americas and Europe, they showed an effect on strains of *S. aureus* and *S. epidermidis* [35]. Another study reported that Kenyan propolis showed differences in the antibacterial activity against *S. aureus* in three studied geographical areas [36]. In this context, we agree with the different authors who found a great variety of propolis that present a diversity of activity against Staphylococci in distinct regions around the world; these investigations have been important for the study of infections caused by this bacterial genus. However, in these studies, the chemical components of the propolis were not described, so the adequate and standardized use of propolis cannot yet be achieved [6].

Table 1. Effect of propolis from several parts of the world on various *Staphylococcus* species.

Propolis Origin	Bacterial Species	Activity	Ref.
Brazil (red, green, and brown propolis from different regions)	*S. aureus*	Red extracts showed highest activity compared with green and brown extracts (MIC: 25–200 μg/mL both propolis)	[30]
Poland	Twelve MSSA and MRSA clinical isolates	Displayed variable effectiveness against twelve clinical isolates (MBC: 0.78–3.13 mg/mL)	[32]
Germany, Ireland, and Czech Republic	*S. aureus*, MRSA, and *S. epidermidis*	The three propolis showed moderate antibacterial activity (MIC: 0.08–2.5 mg/mL)	[28]
Italy	*Staphylococcus* spp. and *S. aureus*	Propolis (MIC: 0.31–2.5 mg/mL) showed inhibitory action on the lipase activity of 18 *Staphylococcus* spp. and an inhibition on coagulase of 11 *S. aureus* strains; showed inhibition of adhesion and consequent biofilm growth of *S. aureus*	[33]
Serbia (53 samples from different regions; blue and orange propolis)	*S. aureus*	All propolis samples showed antibacterial activity, with orange-type (0.1–14.7 mg/mL) showing higher activity than blue-type propolis samples (1.8–12.9 mg/mL)	[34]

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

As propolis functions to support the sterility and health of the beehive, the protective properties of the bioactive compounds in propolis can provide significant benefits for human health [37,38]. In this context, the flavonoids and esters of phenolic acids present in propolis are habitually the active components related to antibacterial effect [39]. Samples of distinct types of propolis from diverse regions in Brazil were studied (red, green, and brown), showing distinct antibacterial activities against different microorganisms, including *S. aureus*. Ferulic acid, gallic acid, caffeic acid, coumaric acid, *p*-coumaric acid, catechin, drupanin, kaempferide, artepillin C, luteolin, and pinocembrin have been identified in propolis, the researchers concluding that Brazilian propolis have various compounds, which present antibacterial activities that could be used for the elaborate of new medicines [40–42]. Similarly, the antibacterial activity of 20 Polish propolis obtained from distinct areas and 5 propolis from agricultural localities were studied. These samples showed distinct antibacterial activity toward *S. aureus* and *S. epidermidis*. For the 20 clinical isolates of *S. aureus* (16 MSSA and four MRSA), the propolis samples presented different activities, two of which showed higher antistaphylococcal activity, probably because these two samples contain more flavonoids that the other samples of propolis studied. The propolis originating from agricultural areas in Southern Poland presented a higher content of bioactive components (different flavonoids and phenolic acids). The samples of Polish propolis effectively eradicated staphylococcal biofilm, suggesting that the identified components are essential for the antibacterial effect of propolis [27,43]. Pinocembrin, galangin, and chrysin identified in South African propolis are known to possess antibacterial activity; combinations of these three flavonoids presented higher inhibition than flavonoids alone against different bacterial strains, including *S. aureus*. These flavonoids showed a synergistic effect to obtain a better antibacterial activity (Table 2) [44]. Propolis from Northern Morocco showed inhibitory effects against *S. aureus*, with the authors identifying different phenolic compounds such as caffeic acid, *p*-coumaric acid, ferulic acid, naringenin, pinocembrin, chrysin, galangin, pinobanksin, and quercetin [45]. We also agree with the studies that have reported the different propolis around the world presenting antistaphylococcal activity; these investigations have described the main active components of propolis and their activity against staphylococci, alone or in combination, which is of relevance for future research [44], since the search for more compounds and better combinations is necessary to treat infections occasioned by the *Staphylococcus* genus.

In another study, synergistic interactions were reported regarding combinations of Irish propolis and antibiotics (two-drug combinations: vancomycin, oxacillin, and levofloxacin) against different microbial pathogens, including MRSA. The authors concluded that the propolis from Ireland increased the synergistic effect and the effectiveness of antibiotics, mainly of vancomycin and oxacillin, that interact on cell-wall synthesis on drug-resistant bacteria [28]. In 2019, Grecka et al. observed the synergistic antistaphylococcal effect against *S. aureus* of one sample of Polish propolis combined with different drugs and fusidic acid; notably, all these drugs present an inhibitory action on protein synthesis [27]. Similarly, the activity of the combination of propolis from Poland with 10 antibiotics against staphylococci on *S. aureus* clinical isolates was proven, suggesting that the combinations of Polish propolis with different drugs potentiated the antibacterial effect on the various strains; however, no synergism was observed in the case of ciprofloxacin and chloramphenicol [32]. Likewise, the synergetic effect of propolis from Italy with some antibiotics on different bacterial strains was assessed, including *S. aureus* and *S. epidermidis*, reporting that Italian propolis enhanced the antibacterial activity of six different antibiotics [33].

Recently, Malaysian propolis and propolis nanoparticles (prepared with Malaysian propolis) exhibited antibacterial and antibiofilm properties against *S. epidermidis.* Propolis nanoparticles drastically inhibited biofilm growth by *S. epidermidis* and reduced the viability of biofilm bacteria compared with propolis extract. Propolis nanoparticles treatment showed significant disruption of biofilm and partial disruption by Malaysian propolis extract, decreasing bacteria in the biofilm. The gene expression in the tested bacteria described that genes related in intercellular adhesion (*IcaABCD*, *embp*) were downregulated by

propolis nanoparticles. Propolis nanoparticles presented a synergistic effect with different drugs, suggesting efficient treatment. The authors concluded that propolis nanoparticles are more efficient than propolis extract alone in inhibiting bacterial biofilms by produce membrane alteration and reducing biofilm growth (Table 3) [46]. Notably, drug-resistant bacteria significantly affect health systems at present; in this context, studies with propolis and its potential antibacterial activity are promising. We agree with the different studies that have demonstrated the effectiveness of propolis, its bioactive components, and the combinations with various antibiotics against the *Staphylococcus* genus. These new sources of natural products are not aimed to replace antibiotic treatment but could be a complement in the treatment of these pathogens that now have resistance to antibiotics [47].

Table 2. Antibacterial effect of diverse propolis and its chemical composition.

Propolis/Compound	Bacterial Species	Identified Compounds	Activity	Ref.
Brazil (red, green, and brown propolis from diverse regions)	*S. aureus*	Ferulic acid, gallic acid, caffeic acid, coumaric acid, *p*-coumaric acid, catechin, drupanin, kaempferide, artepillin C, luteolin, and pinocembrin	All propolis showed distinct antibacterial activities (200–1600 μg/mL)	[40–42]
Poland (25 different samples)	*S. aureus* and *S. epidermidis*; 16 MSSA and four MRSA clinical isolates	Flavonols, flavones, flavanones, pinocembrin, chrysin, pinobanksin, apigenin, kaempferol, *p*-coumaric acid, ferulic acid, and caffeic acid	All propolis (1–8 mg/mL) showed distinct antibacterial effect on *S. aureus* and *S. epidermidis*; in the clinical isolates, all the samples of propolis presented different activities and two of them showed higher antistaphylococcal activity; propolis effectively eradicated staphylococcal biofilm	[27,43]
Pinocembrin, galangin, and chrysin (South African propolis)	*S. aureus*	Pinocembrin, galangin, and chrysin	The combinations of these three flavonoids presented higher inhibition than alone flavonoids (0.04–0.26 mg/mL)	[44]

Table 3. Effects of propolis in combination with various drugs on different bacterial species.

Propolis Origin	Antibiotics	Bacterial Species	Activity	Ref.
Ireland	Two-drug combinations: vancomycin, oxacillin, and levofloxacin	MRSA	Propolis (MIC: 0.4–5 mg/mL) synergistically enhanced the efficacy of antibiotics, especially those acting on cell wall synthesis (vancomycin (0.2 mg/mL) and oxacillin (12.5 mg/mL)) on drug-resistant bacteria	[28]
Poland	Amikacin, kanamycin, gentamycin, tetracycline, and fusidic acid	*S. aureus*	Propolis (16–32 μg/mL) showed a synergistic effect in combination with various antibiotics (1–0.0312 μg/mL) that inhibit protein synthesis	[27]
	Cefoxitin, clindamycin, tetracycline, tobramycin, linezolid, trimethoprim + sulfamethoxazole, penicillin, and erythromycin	*S. aureus* clinical isolates	The combination of propolis (MIC: 0.39–0.78 mg/mL) with different drugs potentiated the antibacterial effect of eight antistaphylococcals (1–30 μg/mL) against all strains	[32]

Table 3. *Conts.*

Propolis Origin	Antibiotics	Bacterial Species	Activity	Ref.
Italy	Ampicillin, gentamycin, streptomycin, chloramphenicol, ceftriaxone, and vancomycin	*S. aureus* and *S. epidermidis.*	Propolis increased the antibacterial effect of ampicillin (0.05–3.12 μg/mL), gentamycin (0.05–1.56 μg/mL), and streptomycin (0.05–50 μg/mL); moderately for chloramphenicol (0.05–25 μg/mL), ceftriaxone, and vancomycin (0.39–3.15 μg/mL)	[33]
Nanoparticles prepared with Malaysian propolis	Rifampicin, ciprofloxacin, vancomycin, and doxycycline	*S. epidermidis*	Propolis nanoparticles (15.63–125 μg/mL disrupted bacterial biofilms by causing membrane damage and significantly reducing biofilm formation, and showed synergism with antibiotics (0.2–25 μg/mL)	[46]

MIC: minimum inhibitory concentration.

2.2. Streptococcus Infections

The genus *Streptococcus* is classified as Gram-positive and catalase-negative, appearing as cocci in pairs and chains on Gram stains. When grown on blood agar, they appear as small colorless colonies that cause beta or complete hemolysis [48]. The species of this genus are the cause of a large number of diseases in the human population, from acute to chronic infections with a wide array of manifestations in both adults and children [49]. In this genus, we can highlight *S. pyogenes, S. pneumoniae*, and *S. mutans*.

Three propolis samples of different geographic origins (Germany, Ireland, and Czech Republic) present moderate antibacterial effect on *S. pyogenes* and *S. pneumoniae* [28]. Similarly, Italian propolis showed antibacterial activity on different clinically isolated Gram-positive strains, including *S. pneumoniae* [33]. Similarly, a sample of Mexican propolis presented antibacterial activity against different microorganisms, including *S. mutans*; compounds such as pinocembrin, chrysin, galangin, alpinetin, dillenetin, isorhamnetin, ferulic acid, syringic acid, and caffeic acid were identified in the propolis. Several compounds (galangin, ferulic acid, syringic acid, and caffeic acid) also showed antibacterial activity against this oral pathogen [50]. In another study, the antibacterial effect of various samples of South Brazilian propolis was assessed: all showed activity against different bacterial strains, including *S. mutans*. All samples of propolis have an inhibitory action on *S. mutans* biofilm growth. In all these samples, diverse compounds were described, concluding that South Brazilian propolis could be an important resource of active components with properties for use in the pharmaceutical sector [42]. In similar research, the antibacterial and antibiofilm activities of propolis from Iran and its main compound, quercetin, were described on different bacterial strains, including *S. mutans* and *S. pneumoniae*, suggesting that Iranian propolis and quercetin were effective on the different bacteria studied and showed an inhibitory activity *S. mutans* biofilm adherence (Table 4) [51]. Several investigations have studied the activity of propolis and some of its bioactive compounds against the genus *Streptococcus*; although the results are encouraging, a limitation of these studies is that the possible mechanisms of action must be studied in vitro and in models [52], which would help to better understand this type of infection and its possible complementary treatments.

Interactions were reported regarding combinations of Irish propolis and distinct drugs (two-drug combinations: vancomycin, oxacillin, and levofloxacin) against different bacterial strains, including *S. pneumoniae* and *S. pyogenes*. The propolis from Ireland increased synergistic effect and the effectiveness of drugs that interact on cell-wall synthesis (vancomycin and oxacillin) [28].

Table 4. Antistreptococcal activity of diverse propolis and its chemical composition.

Propolis/Compound	Bacterial Species	Identified Compounds	Activity	Ref.
Germany, Ireland, and Czech Republic	*S. pyogenes* and *S. pneumoniae*	N. I.	Moderate antibacterial activity (MIC: 0.6–5 mg/mL)	[28]
Mexico	*S. mutans*	Pinocembrin, chrysin, galangin, alpinetin, dillenetin, isorhamnetin, ferulic acid, syringic acid, and caffeic acid	Propolis (MIC: 125–1024 μg/mL) presented antibacterial activity; galangin, ferulic acid, syringic acid, and caffeic acid showed activity against this oral pathogen	[50]
South of Brazil (different samples)	*S. mutans*	Gallic acid, caffeic acid, coumaric acid, artepillin C, and pinocembrin	All samples of propolis (25–800 μg/mL) have an inhibitory action biofilm growth	[42]
Iran/ quercetin	*S. mutans* and *S. pneumoniae*	Quercetin	Both propolis (MIC: 3.12–100 μg/mL) were efficient against the bacteria studied and showed an inhibitory activity *S. mutans* biofilm adherence	[51]

N.I., none identified; MIC: minimum inhibitory concentration.

2.3. Gastrointestinal Infections

Gastrointestinal infections constitute a great proportion of the acute and chronic disease burden in all the world. Some bacterial, viral, and parasitic microorganisms infect through contaminated food and water or from human to human. The WHO mentions that diarrhea causes 2.2 million deaths each year worldwide (about 4%) [53]. For this reason, the studies examining the effects of propolis on the main bacterial pathogens that cause gastrointestinal diseases are analyzed below.

The propolis of different geographic origins presented a moderate antibacterial effect on *Escherichia coli*, *Salmonella choleraesuis*, and *Shigella flexneri* [28]. In another study, 53 propolis were obtained from various areas of Serbia; the orange-type propolis samples showed higher antibacterial activity against *E. coli, Salmonella enteritidis, S. flexneri,* and *Listeria monocytogenes* [34]. Another study reported that Kenyan propolis showed differences in the antibacterial activity from three different geographical areas against different bacterial strains, including *E. coli* [36].

Similarly, Brazilian propolis (red, green, and brown; collected in diverse regions) as well as Southern Poland propolis showed distinct antibacterial activities against different microorganisms, including *E. coli* and *L. monocytogenes*; bioactive components such as ferulic acid, *p*-coumaric acid, caffeic acid, catechin, luteolin, drupanin, kaempferide, artepillin C, pinocembrin, chrysin, pinobanksin, apigenin, and kaempferol were identified [40,41,43]. In another study, pinocembrin, galangin, and chrysin (principal components South African propolis) were found to possess antibacterial activity against different microorganisms, including *L. monocytogenes* and *E. coli*, and combinations of these three flavonoids presented higher inhibition activity than components alone. The authors observed that these compounds worked synergistically to achieve the best antibacterial effect (Table 5) [44]. Propolis from Northern Morocco showed inhibitory effects against different Gram-negative strains, including *E. coli*; the researchers identified caffeic acid, *p*-coumaric acid, ferulic acid, naringenin, pinocembrin, chrysin, galangin, pinobanksin, and quercetin [45]. As mentioned by different authors who have studied the activity of propolis and some of its bioactive compounds on the effect against pathogens that cause gastrointestinal infections, we agree that the propolis present a great antibacterial diversity; nevertheless, these investigations contribute limited conclusions; therefore, it is necessary to carry out more studies focusing on understanding the antibacterial activity of propolis and trying to find its possible mechanism of action [54]. In addition, it is important to conduct in vivo and clinical trials in

propolis of various areas to consider the differences in the chemical components of each one and, therefore, the different antibacterial activities that it may present [55].

Table 5. Antibacterial activity of different propolis on various microorganism species.

Propolis/Compound	Bacterial Species	Identified Compounds	Activity	Ref.
Germany, Ireland, and Czech Republic	*E. coli*, *S. choleraesuis*, and *S. flexneri*	N.I.	All showed moderate antibacterial activity (MIC: 0.6–5 mg/mL)	[28]
Brazil (red, green, and brown propolis) and Southern Poland	*E. coli* and *L. monocytogenes*	Ferulic acid, *p*-coumaric acid, caffeic acid, catechin, luteolin, drupanin, kaempferide, artepillin C, pinocembrin, chrysin, pinobanksin, apigenin, and kaempferol	All samples showed distinct antibacterial activities (25–800 μg/mL)	[40,41,43]
Pinocembrin, galangin, and chrysin (South African propolis)	*L. monocytogenes* and *E. coli*	Pinocembrin, galangin, and chrysin	The combinations of these three flavonoids (0.04–0.26 mg/mL) presented higher inhibition activity than alone components	[44]

N.I., none identified; MIC: minimum inhibitory concentration.

Another research showed that Brazilian propolis presents a bacteriostatic effect on *Salmonella typhi*, and Bulgarian propolis presented a bactericidal effect and a synergism with chloramphenicol, tetracycline, and neomycin (act on the ribosome) on this same pathogen [56].

2.4. Nosocomial Infections

Nosocomial infections are not commonly found when admitted to hospital or are probably incubating. These infections are typically contracted in the hospitalization and generally manifest 48 h after [57]. The numbers of these infections are worrying: according to estimated figures from the CDC, in 2014, 11,282 patients suffered from healthcare-associated infections in USA hospitals alone. The main infections encompass primary bloodstream infection, surgical site infections, pneumonia, and urinary tract infections [58]. In this area, *Haemophilus influenza, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* are the cause of most of respiratory and renal nosocomial infections, respectively.

The antibacterial effect of Brazilian propolis (red, green, and brown) of various areas was studied; the red extracts demonstrated higher activity than green and brown extracts against different bacterial species, including *Klebsiella* sp. [30]. Similarly, a study reported the moderate antibacterial effect of propolis of distinct geographic regions (Germany, Ireland, and Czech Republic) on *P. aeruginosa, H. influenzae, K. pneumoniae*, and two clinical isolates of *K. pneumoniae* [28]. Another studies reported that Cameroonian, Congolese, and Kenyan propolis showed differences in antibacterial activity against various microorganisms, including *K. pneumoniae* and *P. aeruginosa* (Table 6) [35,36]. The greatest limitation of these investigations is that they remained at a qualitative level, only describing whether or not propolis presented activity; they did not mention any possible mechanism of action of propolis against these pathogens, which is essential to better understanding nosocomial infections and how to combat them [59,60].

Another study mentioned that various South Brazilian propolis showed activity against different bacterial strains, including *P. aeruginosa*. In all samples, gallic acid, caffeic acid, coumaric acid, artepillin C, and pinocembrin were identified [42]. Propolis originating from Southern Poland showed stronger antibacterial activity against different microorganisms, including *K. pneumoniae* and *P. aeruginosa*. Additionally, pinocembrin, chrysin, pinobanksin, apigenin, kaempferol, *p*-coumaric acid, ferulic acid, and caffeic acid were identified [43]. In other research, the effects of Albanian propolis were evaluated in various virulence factors of *P. aeruginosa*. Propolis inhibited the microbial development and biofilm

growth; also, propolis decreased extracellular DNA release and phenazine production. Compounds were identified in the propolis, such as caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, quercetin, apigenin, pinobanksin, chrysin, pinocembrin, galangin, and caffeic acid phenethyl ester (CAPE), with the authors including that Albanian propolis contains different components with activity on biofilm-related infections [61]. In another investigation, combinations of pinocembrin, galangin, and chrysin (principal components of South African propolis) showed a better inhibitory effect than single compounds against different bacterial strains, including *P. aeruginosa* and *K. pneumoniae*, suggesting that these compounds present a synergistic interaction favoring antibacterial activity (Table 7) [44]. Likewise, propolis from Northern Morocco showed inhibitory effects against different Gram-negative strains, including *P. aeruginosa*. Different phenolic compounds, such as caffeic acid, *p*-coumaric acid, ferulic acid, naringenin, pinocembrin, chrysin, galangin, pinobanksin, and quercetin, were identified from the propolis [45]. We consider the studies on propolis and some of its bioactive compounds against pathogens that cause nosocomial infections to be of relevance, since they mentioned a possible mechanism of action [62], although more studies are needed related to this area. It is also necessary to carry out research using in vivo models and then to clinical trials to help knowledge possible via action of propolis and be able to combat this type of infection [63,64].

Table 6. Antibacterial activity of diverse propolis on different microorganisms that cause nosocomial infections.

Propolis Origin	Bacterial Species	Activity	Ref.
Brazil (red, green, and brown propolis from different regions)	*Klebsiella* sp.	Red extracts showed higher activity than green and brown extracts (MIC: 31.1–1000 μg/mL both propolis)	[30]
Germany, Ireland, and Czech Republic	*P. aeruginosa*, *H. influenzae*, *K. pneumoniae*, and two clinical isolates of *K. pneumoniae*	All propolis showed moderate antibacterial activity (0.06–2.5 μg/mL)	[28]
Cameroon, Congo, and Kenya	*K. pneumoniae* and *P. aeruginosa*	All propolis showed differences in antibacterial activity (50 mg/mL)	[35,36]

MIC: minimum inhibitory concentration.

Table 7. Effect of diverse propolis and its main identified components on *P. aeruginosa* and *K. pneumoniae*.

Propolis/Compound	Bacterial Species	Identified Compounds	Activity	Ref.
Albania	*P. aeruginosa*	Caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, quercetin, apigenin, pinobanksin, chrysin, pinocembrin, galangin, and CAPE	Propolis (15.6–62.5 mg/mL) inhibited the microbial development and biofilm growth, also decreased extracellular DNA release and phenazine production	[61]
Pinocembrin, galangin, and chrysin (South African propolis)	*P. aeruginosa* and *K. pneumoniae*	Pinocembrin, galangin, and chrysin	Combinations of the three flavonoids (0.6–25 μg/mL) present better antibacterial effect than single components	[44]

Another study reported that two-antibiotic combinations (vancomycin, oxacillin, and levofloxacin) and Irish propolis showed synergism on *H. influenzae*, concluding that the propolis from Ireland increases the synergism and effectiveness of vancomycin and oxacillin, which act on cell wall synthesis [28].

As we already mentioned, resistance to antibiotics is a serious health problem, since it makes it difficult to properly treat several diseases of bacterial origin. The documented effects of propolis and its derivatives on bacteria such as MRSA make them ideal candidates for clinical studies in order to evaluate their effectiveness on antbiotic-resistant bacterial dis-

eases. The clinical application of propolis should not focus on the substitution of antibiotics, but on complementing and improving the efficacy of these when co-administered.

3. Antifungal Activity of Propolis

Fungal infections are responsible for over one million human deaths annually and are an increasingly important cause of mortality and morbidity [65]. In recent years, fungal infections have increased significantly, being considerably high in immunosuppressed patients [66]. Unfortunately, the low number of available treatments and the misuse of the antifungal medications have led to the selection of resistant microorganisms [67], which is why the search for new, effective, and inexpensive antifungal agents is crucial to overcoming existing resistance mechanisms [66]. Different natural products from distinct places and latitudes come to constitute a little-explored group of agents with antifungal capacity; of all these, propolis has special relevance [66], as recent studies have evaluated it as a natural product with potential for the development of antifungal drugs without toxicity [68,69].

3.1. Candidiasis

The genus *Candida* is a group of fungi known for their dimorphic capacity and is commonly isolated from the microbiome of healthy individuals (intestinal tract, oral cavity, skin, and vaginal cavity) [70–72]. However, when the host's immunity becomes compromised by diseases such as HIV, AIDS, cytotoxic therapies, uncontrolled diabetes mellitus, or people of very young or very old age, *Candida* can behave like a pathogenic fungus. The progressive increase in the number of infections caused by *Candida* worldwide has increased in recent decades; this may be due to the significant increase in the population at risk, particularly the spread of HIV, immunosuppressive therapies, and the increase in the use of permanent devices [72–74]. The different species of *Candida* were classified as the fourth main agent that generate highly relevant infections worldwide; the magnitude of these diseases worldwide is alarming [72,75], and it has been recorded that infections caused by Candida species have a high crude mortality rate, exceeding the number caused by *S. aureus* and *P. aeruginosa* in nosocomial infections of the bloodstream [76]. It is important to highlight that the incidence in the annual rates of nosocomial infections of the bloodstream caused by Candida at the beginning of the century presented a variability of 6.0 to 13.3 and from 1.9 to 4.8 cases per 100,000 inhabitants in the United States and Europe, respectively [77–79]. Hence, *Candida* is a healthcare priority, and new antifungal therapeutic approaches are urgently needed. The propolis from different geographical regions has demonstrated anti-*Candida* activity, as described below.

The distinct clinical isolates of different species of the genus *Candida* extracted from vaginal exudates of patients with vulvovaginal candidiasis were completely suppressed by Brazilian propolis with a very small variation independent of the yeast species [80]. Likewise, Brazilian green propolis showed the ability to suppress the growth and biofilm formation of vaginal isolates of *C. albicans* [81]. In another research, the fungicidal effect of Brazilian propolis was demonstrated on three morphogenetic types of *C. albicans*, and the induced cell death was mediated by metacaspase and Ras signaling. This was corroborated by propolis inhibiting yeast transformation to hyphal growth. Moreover, a topically applied pharmaceutical formula based on propolis can partially control *C. albicans* infections in a vulvovaginal candidiasis infection in a mouse model [82].

Within Europe, the antifungal effect of different propolis has been investigated. The effect of four different Polish propolis samples on azole-resistant *Candida* clinical isolates was studied, with only one of the four propolis samples revealing high antifungal activity [83]. Similarly, Portuguese and French propolis presented distinct antifungal activities against *C. albicans* and *C. glabrata* [31,84]. The antifungal effect of propolis obtained in distinct geographical areas of the European continent was investigated. All propolis used reported an antifungal effect both in reference strains and different species of the *Candida* genus from clinical isolates. Propolis from Ireland and Czechia showed very good fungicidal effects,

while propolis from Germany showed mostly fungistatic activity; *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* were the most sensitive *Candida* [28].

In Asia in 2020, Alsayed et al. reported a fungicidal effect of propolis from Saudi Arabia against *C. zeylanoides*, *C. famata*, *C. sphaerica*, *C. guilliermondii*, *C. magnoliae*, and *C. colliculosa* and a fungistatic effect against *C. krusei*, *C. pelliculosa*, and *C. parapsilosis* [85]. In other study, propolis from Turkey showed antifungal activity against different clinical isolates of *Candida* [86]. Similarly, the antifungal effect of aqueous and ethanolic extracts of Iranian propolis was described against *Candida* samples that were collected from 23 oral cavities of patients presenting candidiasis in the oral cavity (isolating 22 samples of *C. albicans* and one *C. glabrata*). Both extracts of Iranian propolis demonstrated inhibitory effects on *Candida*, but the extract that presented greater effectiveness even above the aqueous one was the ethanolic [87]. Other researchers evaluated the antifungal activity of propolis and propolis-loaded nanoparticles (EEP-NPs) from Thailand, observing the impact they have on specific factors that contribute to the pathogenesis of *C. albicans*, where EEP-NPs were mostly active compared to propolis in its free form, inhibiting virulence factors such as adhesion, hyphal germination, biofilm formation, and invasion. It should be noted that the EEP-NPs showed a decrease in the expression of genes related to adhesion processes linked to the hyphae of *C. albicans*, demonstrating that the EEP-NPs have the ability to mediate a great anti-*Candida* activity, attacking key factors of virulence, such as the inhibition of the expression of genes related to adhesion-related proteins, which mediate the morphological change of *C. albicans*, attenuating the virulence of the yeast (Table 8) [88]. In Africa, few studies were found for this review. One was conducted by Papachroni et al. in 2015, who analyzed four propolis of distinct areas of Africa, which showed an effect on *C. albicans*, *C. tropicalis*, and *C. glabrata* [35]. We think that the aforementioned studies that have demonstrated the anti-*Candida* activity of propolis have a significant impact on this issue, since most of them reported fungicidal or fungistatic activity exhibited by propolis from different regions on different strains and clinical isolates of *Candida* [89], as well as some possible mechanisms of action through which propolis inhibits this yeast, such as virulence factors that favor the pathogenicity of *C. albicans*. Some studies even reported the activity of propolis being very promising in in vivo models of candidiasis infections; however, the limitation of all these investigations is that they did not mention the composition of the different propolis, since, as we mentioned earlier, it is important to know and determine the active components present in propolis to identify which molecules are responsible for this antifungal effect [90].

Various studies around the world have focused on the search for components in the propolis that have antifungal effect; below, we describe some of the investigations that revealed the antifungal potential of this natural product. A fraction of the Brazilian red propolis rich in benzophenones was analyzed, which showed activity against different clinical isolates of *C. parapsilosis* and *C. glabrata* resistant to antifungal agents, like fluconazole [91]. Equally, other propolis from Brazil analyzed by different researchers showed fungicide action on different strains, with *C. albicans* being more sensitive and *C. parapsilosis* being the most resistant strain studied. An in vivo study described that gels based on propolis had an antifungal effect similar to clotrimazole cream. In this propolis, different compounds were identified [92]. In Europe, one of the most extensive studies of propolis extracts was conducted, analyzing the effects of 50 different propolis extracts from Polish hives against 89 *Candida* spp. clinical isolates. Most of the samples of propolis produced satisfactory activity, showing high activity in the inhibition of biofilm formation generated by *C. glabrata* and *C. krusei* on the surfaces of polyvinyl chloride and silicone catheters. The propolis inhibited the yeast-to-mycelia morphological change and mycelial growth of *C. albicans*. In addition, the propolis combined with fluconazole or voriconazole on *C. albicans* was shown to have a clear synergism. The chemical composition of three propolis with high and one with low antifungal effect was determined (finding different flavonoids and phenolic compounds), providing evidence that the fungal cell membrane could be the target of propolis [66]. Similarly, in 2019, Pobiega et al. analyzed different Polish propolis (agricultural regions and Southern Poland), the latter being noted by greater antifungal

activity against different microorganisms, including *C. albicans* and *C. krusei*, also showing a higher content of bioactive components (Table 9) [43]. Within Africa, samples providing satisfactory results were reported: one of them was Egyptian propolis, which presented antifungal activity against *C. albicans*. In addition, the authors identified compounds such as ferulic acid, cis- and trans-caffeic acids, pinostrobin, and galangine, among others [93]. We agree that the identification of the composition of propolis from distinct areas of the world is crucial, which provides an approach to elucidating some bioactive compounds with antifungal activity and thus paving the way for future research, for example, to complement antifungal drugs with propolis or with its bioactive compounds. However, in the aforementioned studies, the mechanism of action by which propolis or any of its identified molecules exerts their antifungal effect on the different strains of *Candida* must be further investigated [94]. In addition, this pathogen is the cause of many infections in which alternative treatment is needed; propolis could be a promising option.

Table 8. Anti-*Candida* activity of different propolis around the world.

Propolis Origin	Fungal Species	Activity	Ref.
Brazil (green propolis)	Vaginal isolates of *C. albicans*	Suppress growth and biofilm formation	[81]
Brazil	*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. guilliermondii*, and *C. parapsilosis*	All strains were suppressed, with minimal variation independent of the yeast species (273.43 μg/mL)	[80]
	C. albicans	Showed fungicidal activity against the three morphogenetic types; the induced cell death was mediated by metacaspase and Ras signaling	[82]
Brazil (topical pharmaceutical preparation based upon propolis)	Vulvovaginal candidiasis infection in a mouse model (*C. albicans*)	Can partially control *C. albicans* infections (0.05%, 0.1%, and 0.2%)	
Poland (different samples)	Azole-resistant *C. albicans*, *C. glabrata*, and *C. krusei* clinical isolates	Only one of the four propolis samples revealed high activity (MFC: 0.0006–1.25% *v*/*v*)	[83]
Portugal and France	*C. albicans* and *C. glabrata*	Presented distinct antifungal activities (15.63–250 μg/mL)	[31,84]
Germany, Ireland, and Czech Republic	*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*	Propolis from Ireland and Czech demonstrated excellent fungicidal (0.1–5 mg/mL) effects; propolis from Germany showed mostly fungistatic (0.1–2.5 mg/mL) activity. *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* were the most sensitive.	[28]
Saudi Arabia	*C. zeylanoides*, *C. famata*, *C. sphaerica*, *C. guilliermondii*, *C. magnoliae*, *C. colliculosa*, *C. krusei*, *C. pelliculosa*, and *C. parapsilosis*	Showed fungicidal (2.5% *v*/*v*) and fungistatic (5%) effects against different strains	[85]
Iran	22 samples of *C. albicans* and one sample of *C. glabrata* isolates from oral cavities of patients with clinical oral candidiasis	Both extracts showed inhibitory effects on *Candida*, but the extract that presented greater effectiveness even above the aqueous (0.2–130 mg/mL) one was the ethanolic (0.4–210 mg/mL)	[87]
Propolis-loaded nanoparticles from Thailand	*C. albicans*	Inhibited the virulence factors of *C. albicans*, such as adhesion, hyphal germination, biofilm formation, and invasion (1 and 2 mg/mL)	[88]

MFC: medium fungicidal concentration.

Table 9. Antifungal activity of diverse propolis and its chemical composition on different *Candida* strains.

Propolis Origin	Fungal Species	Identified Compounds	Activity	Ref.
Brazil	*C. albicans* and *C. parapsilosis*	Caffeic acid, *p*-coumaric acid, cinnamic acid, aromadendrin, artepillin C	Showed fungicide (0.5%) action on different strains; *C. albicans* being the most sensitive, and *C. parapsilosis* being the most resistant	[92]
Brazil (propolis-based gels)	Vaginal candidiasis mouse model		Presented antifungal effect (1%) similar to clotrimazole cream	
Poland (50 different samples)	69 *C. albicans*, 10 *C. glabrata*, and 10 *C. krusei* clinical isolates	Caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, pinobanksin, luteolin, kaempferol, apigenin, pinocembrin, acacetin, galangin, kaempferide, and naringenin	All samples showed high activity in the inhibition of biofilm formation by *C. glabrata* and *C. krusei* (0.04–1.25% *v*/*v*); inhibited yeast-to-mycelia morphological change and mycelial growth of *C. albicans* (0.16–0.31% *v*/*v*); propolis combined with fluconazole and voriconazole on *C. albicans* (0.5–512 μg/mL) was shown to have a clear synergism	[66]
Poland (from agricultural areas and Southern Poland)	*C. albicans* and *C. krusei*	Pinocembrin, chrysin, pinobanksin, apigenin, kaempferol, *p*-coumaric acid, ferulic acid, and caffeic acid	Samples from Southern Poland showed greater antifungal activity (2–16 mg/mL)	[43]

3.2. Trichophyton Infections

The diseases known as dermatophytoses are mycoses generated by fungi that commonly cause different infections in the superficial epithelia in animals and principally in humans. Various filamentous fungi are the cause of these diseases that can invade and acquire nutrients from keratinized epithelia (skin, hair, and nails) [95,96]. Dermatophytoses have the ability to affect people all over the planet, having a higher incidence level in hot tropical countries with high humidity. Approximately 10% to 15% of people are infected by dermatophytes at some time in their life [97]. It is known that dermatophytoses have the ability to affect approximately 25% of the world population according to data from WHO, as well as to generate adults carrying the disease with completely asymptomatic characteristics in a percentage ranging from 30% to 70% [98]. In developed countries, dermatophytes are the main causes of onychomycosis identified with a frequency ranging from 80% to 90%. Around the world, the prevalence of tinea pedis is has been reported approximately at 5.5%, representing 50% of all cases of nail disease [99]. The main cause of these diseases is the genus *Trichophyton*.

The Brazilian Amazon rainforest is a huge source of plant biodiversity, which is why the propolis derived from this area has many biological properties, one of which is the antifungal activity described by Silva et al. in 2015, who stated that red and green propolis are active against strains of *T. rubrum*, *T. tonsurans*, and *T. mentagrophytes*, with red propolis being more efficient than green [100]. Similarly, Brazilian green propolis showed antifungal activity against the preformed biofilms of two clinical isolates of *Trichophyton* from onychomycosis cases, the authors observing that the total biomass and the percentage of living cells of the biofilms that were subjected to the treatment with propolis were lower than in the control for both isolates; therefore, Brazilian propolis had the ability to decrease the number of cells in the preformed *Trichophyton* biofilm. Sixteen patients infected of onychomycosis were treated with topical propolis twice a day, with a 6-month follow-up period. After treatment, the data obtained were encouraging, observing a mycological and clinically total resolution in the nails and showing a complete improvement of the natural morphology of the nail and the disappearance of the fungus of up to 56.25% of patients.

Brazilian propolis is a therapy drug with great potential to be used to topically treat onychomycosis caused by *Trichophyton* [101]. Likewise, the Portuguese propolis presented distinct antifungal activity against *T. rubrum* (Table 10) [84]. The different previous studies showed that propolis has variable antifungal activity on different *Trichophyton* strains, inhibits the biofilm of clinical isolates, and a topical treatment based on propolis improved onychomycosis in patients; however, there are several limiting factors in the research of the activities of propolis against this fungus. One of the recurring omissions in this type of research is to omit to description of the components of propolis, since this is transcendental for identifying the bioactive components. Future studies must search for a possible mechanism of action against this pathogen, as it causes very common infections; therefore, it is important for there to be accessible options or traditional medicine to treat them, since a large part of the population uses this type of treatment. In addition, it is vitally relevant to carry out clinical studies that help to validate the distinct doses of propolis that help the treatments, because variety in active principles and the biomedical effects of the several propolis have to be taken into account [102].

Table 10. Effect of propolis on different species of *Trichophyton*.

Propolis Origin	Fungal Species	Activity	Ref.
Brazil (red and green propolis)	*T. rubrum*, *T. tonsurans*, and *T. mentagrophytes*	Both propolis were active on the strains, but the red propolis was more efficient than the green (256–1024 μg/mL)	[100]
Brazil (green propolis)	Clinical isolates of *T. rubrum* and *T. interdigitale* from onychomycosis cases	Propolis had the ability to decrease the cells in the preformed *Trichophyton* biofilm (0.044–0.088 mg/mL)	[101]
Brazil (topical green propolis treatment)	Sixteen patients with onychomycosis	Treatment showed a complete mycological and clinical cure of onychomycosis (10%)	

3.3. Aspergillus and Penicillium Infections

Some species of the *Aspergillus* genus are responsible for chronic pulmonary Aspergillosis (CPA) disease, which can range from nonprogressive to severe effects, such as chronic necrotizing pulmonary Aspergillosis [103]. The number of people around the world who have CPA is estimated at three million, and it is believed that Asia has the highest number of disease cases in comparison to other continents [104]. For the year 2019, it was calculated that after pulmonary tuberculosis, 12 million patients developed CPA [105]. In addition, the species of the genera *Aspergillus* and *Penicillium* produce various secondary metabolites known as mycotoxins [106]. In this set of toxins, we find the aflatoxins, fumonisins, deoxynivalenol, ochratoxin A, and zearalenone are agriculturally important and dietary mycotoxins exposure is associated with many chronic health risks, such as cancer, immune suppression, digestive, blood, and nerve defects [107,108]. Next, studies performed with propolis and these species give these fungal gears are described.

Portuguese propolis presented varying antifungal activity against *Aspergillus fumigatus* [84]. Propolis from different latitudes can present very similar biological activities, as in the case of United States and Chinese propolis against *Penicillium notatum*. With both propolis, the structure and morphology of hyphae were damaged, inhibiting the development of mycelium. Propolis treatment raised extracellular conductivities, showing that propolis probably affects the cell membrane. In addition, a decrease in the activity of enzymes related to the functioning of cellular respiration of *P. notatum* (succinate dehydrogenase and malate dehydrogenase) was observed. Additionally, quantitative proteomic analysis (iTRAQ-based) related to energy metabolism and sterols biosynthetic pathway of *P. notatum* in presence the propolis was described, which showed that 88 proteins (25.8%) were upregulated and 253 (74.2%) were downregulated, of a total of 341 proteins. The major compounds

in both propolis were pinocembrin, pinobanksin-3-O-acetate, galanin, chrysin, pinobanksin, and pinobanksin-methyl ether. The authors suggest that all these different properties that propolis has on *P. notatum* can interfere with its development [109]. Similarly, Southern Poland propolis showed antifungal activity against different microorganisms, including *A. niger* and *A. ochraceus*, and were found to contain pinocembrin, chrysin, pinobanksin, apigenin, kaempferol, *p*-coumaric acid, ferulic acid, and caffeic acid [43]. Few studies have been conducted on the effects of propolis against the genus *Aspergillus*, indicating a large gap in the literature. However, the previous studies only focused on observing whether propolis has antifungal activity; only the identified compounds were mentioned, and no correlation was mentioned between the activity and the propolis components, so research is lacking on this topic [110]. We note that study by Xu et al. provides a clear example of how to study natural products on different microorganisms to determine if they present activity or not, to later identify the chemical composition, and then try to find a possible mechanism of action by which natural products could inhibit the pathogen. Finally, the next step in Xu et al.'s research is to conduct studies on in vivo models and clinical trials to provide an alternative and complementary treatment for fungal infections.

Currently, some antifungal drugs are available; however, the problem in treating these diseases is the toxicity toward the host or the emergence of drug resistance in pathogen populations. Here, we describe the efficacy of propolis against different fungal pathogens, where the effect of propolis has been demonstrated in vitro and in vivo [111], as well as in different pathogenicity mechanisms; in some cases, an activity similar to that of the drugs used to treat mycoses has been reported. One of the most remarkable aspects is the use of propolis in clinical studies, where it was shown that propolis can be an alternative that complements the treatment of some of these diseases [112], so it is important to realize more clinical trials to support the effectiveness of propolis in addition to implementing trials focused on evaluating toxicity to determine a standardized dose of propolis that is safe for consumption and application in humans, as well as study the components of each propolis used. These studies could give scientific support to natural products widely used as a therapeutic alternative in rural communities and in developing nations.

4. Antiparasitic Activity

Parasitic diseases continue to take an enormous toll on human health globally, particularly in tropical regions [113,114]. Intestinal and protozoan infections are the most common parasitic diseases. Protozoan parasites are unicellular eukaryotes responsible for 1.3 million deaths worldwide annually [114,115]. In several countries, these diseases are unfortunately not a priority with respect to their surveillance, prevention, and treatment. Among these diseases are malaria, Chagas disease, leishmaniasis, trichomoniasis, amebiasis, and giardiasis [116–118]. Below, we discuss the studies that have been conducted with propolis on the pathogens that cause these diseases.

4.1. Malaria

Malaria is a disease that can cause death generated by a protozoan parasite of the Plasmodium genus. This disease is transmitted by female Anopheles mosquito bites. It is estimated to impair about 219 million people each year in 87 countries, mainly affecting pregnant women and children aged between 0 and 5 years [119]. Propolis has been used from some countries to study its antimalarial effects on species of the genus Plasmodium. In an effort to find alternatives for this disease, 20 propolis from different provinces in Cuba were evaluated in vitro, with three showing significant activity on *Plasmodium falciparum*. Chemical composition analyzes were carried out for propolis, where compounds of phenolic origin and triterpenes such as linquiritigenin and lupeol were found; these compounds were already reported to have activity against *P. falciparum* [120]. Similarly, twelve propolis from Libya were evaluated in vitro and demonstrated antiprotozoal activity, including against *P. falciparum* [121]. Propolis also showed antimalarial properties in vitro, for example, in the case of four Iranian propolis that showed in vitro and in vivo activity at different

concentrations against *P. falciparum*. The chemical composition of the two extracts with higher activity was determined, and molecules such as palmitic acid, stearic acid, pinocembrin, tectochrysin, and 4′,5-dihydroxy-7-methoxyflavanone with an antiplasmodial effect were identified [122]. Saudi propolis considerably suppressed parasitemia and demonstrated an important effect on decreasing anemia in *Plasmodium chabaudi*-infected mice, reducing oxidative damage by enhancing the catalase function and the glutathione concentrations, and enhancing the quantities of pro-inflammatory cytokines. It is reported that these cytokines promote phagocytosis, chemotaxis, and antibody-dependent cytotoxicity. Furthermore, they are responsible for the activation of neutrophils as well as protection against this parasite (Table 11) [123]. These works demonstrate the promise of using propolis against this complex disease; however, some of these works lacked a chemical analysis of propolis. As mentioned previously, it is necessary that future studies with propolis include the origin and description of the chemical composition [6]. Some of the compounds such as lupeol and liquiritigenin have been reported to have antiplasmodial activity [124,125] and could be related to the effects of Cuba's propolis against this parasite. However, it is still necessary to realize different studies to better understand the effects of propolis in the treatment of this disease.

Table 11. Antimalarial effect of propolis and its chemical composition.

Propolis Origin	Parasitic Species	Identified Compounds	Activity	Ref.
Cuba (20 different samples)	*P. falciparum*.	Liquiritigenin and lupeol	Three samples of propolis (0.2 μg/mL) shows activity against *P. falciparum*	[120]
Libya (12 different samples)		N.I.	All samples of propolis (1.65–53.6 μg/mL) showed antiprotozoal activity	[121]
Iran (four different samples)		Palmitic acid, stearic acid, pinocembrin, tectochrysin, and 4′,5-dihidroxy-7-methoxyflavanone	All samples of propolis presented antimalarial in vitro and in vivo activity at different concentrations (16.2–80 μg/mL)	[122]
Saudi Arabia	*P. chabaudi*-infected mice	N.I.	Considerably suppressed the parasitemia and demosntraed important effect in decresing anemic in infected mice (25–100 mg/Kg), reduced oxidative damage by enhancing the catalase function and the glutathione concentrations, and enhanced the quantity of TNF-α, IFN-γ, G-CSF, and GM-CSF.	[123]

N.I. = none identified.

4.2. Chagas Disease

Trypanosoma cruzi is a protozoan parasite responsible for Chagas disease, which is transmitted mainly by Hematophagous triatomine insects, and to lesser extent by oral, congenital, blood transfusion and organ transplantation [126]. This disease is a problem for most of Latin America and especially affects marginalized zones; it is estimated that 8–10 million people are infected each year [126,127]. Extensive research has been conducted on propolis as an anti-trypanosome agent using in vitro models. We highlight the thorough study on various propolis from Brazil, of which the activity of propolis extract on *T. cruzi* trypomastigotes was reported [30]. Some propolis were effective on the three forms of the parasite, and treatment with propolis strongly inhibited infection levels by promoting lysis of bloodstream trypomastigotes and diminished the number of parasites in peritoneal

macrophages and infected heart muscle cells [128]. Some propolis from Brazil showed an in vitro effect against *T. cruzi*; their chemical composition was determined, and caffeic acid, cinnamic acid, pentenoic acid, ferulic acid, linoleic acid, amyrin, and pinostrobin, amongst others, were identified; however, in these studies, the anti-trypanosomal activity of these compounds was not evaluated [129,130]. Interestingly, other authors reported that the application of natural products obtained from propolis produced anti-trypanosome effects; for example, four components were isolated from Brazilian propolis and two were effective on *T. cruzi* [131]. Similarly, Brazilian and Bulgarian propolis were shown to have activity against this parasite, diminishing replication of the parasite without damaging the membrane of the host cell. Microscopic analysis showed that the main organelles damaged by the extracts were mitochondrion and reservosomes [132]; two Bulgarian propolis share many bioactive compounds, mainly flavonoids and a remarkable antitrypanosomal effect; epimastigotes were more sensitive than trypomastigotes. The efficacy of either of the two Bulgarian propolis on trypomastigotes was similar to that of the reference drug [133].

Despite the encouraging results from the in vitro tests, in vivo studies are scarce. For example, treatment with Bulgarian propolis in *T. cruzi*-infected mice led to a reduction in parasitemia and showed no toxic hepatic or renal effect, the spleen mass decreased, and the initial inflammatory reaction was modulated, favoring a greater number of $CD8^+$ and partially inhibiting the increase in CD4 [134]. Studies conducted with propolis from Brazil in infected mice recorded a decrease in the number of parasites and mortality of the animals without generating toxicity or injury on other tissues, so it could be assayed in combination with other drugs as a potential metacyclogenesis blocker (Table 12) [135].

Table 12. Anti-trypanosome activity of different propolis and its chemical composition.

Propolis Origin	Parasitic Species	Identified Compounds	Activity	Ref.
Brazil	*T. cruzi*	N.I.	Activity on *T. cruzi* trypomastigotes.	[30]
		N.I.	Effective on the three forms of the parasite strongly inhibits infection levels promoting lysis of bloodstream trypomastigotes, diminishing the number of parasites in peritoneal macrophages and infected heart muscle cells (0.1–0.75 mg/mL)	[128]
		Caffeic acid, cinnamic acid, pantenoic acid, ferulic acid, linoleic acid, amyrin, pinostobin	In vitro effect against *T. cruzi* (0.4–1.4 mg/mL)	[129,130]
		3-prenyl-4-hydroxycinnamic acid and 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran.	Propolis (2.64 mg/mL) and its compounds (0.73–1.2 mg/mL) identified showed anti-trypanosome effects	[131]
Brazil and Bulgaria		N.I.	Both propolis (0.015–1.5 mg/mL) showed activity against *T. cruzi*, diminishing the infection and the intracellular replication of amastigotes; in epimastigotes, the main targets are the mitochondrion and reservosomes	[132]
Bulgaria (two different samples)		Caffeic acid, stearic acid, oleic acid, ferulic acid, coumaric acid, pinocembrin, chrysin, pinostrobin	Both samples of propolis had great inhibition effect mainly on *T. cruzi* epimastigotes (48.6–84.8 mg/mL); effect on trypomastigotes (160.5–1065.8 mg/mL) was similar to that of the reference drug	[133]

Table 12. *Conts.*

Propolis Origin	Parasitic Species	Identified Compounds	Activity	Ref.
Bulgaria	*T. cruzi*-infected mice.	N.I.	Reduced parasitemia and showed no toxic hepatic or renal effect, decreased spleen mass, modulated the initial inflammatory reaction, favored a greater number of $CD8^+$, and partially inhibited the increase in CD4 (50mg/Kg)	[134]
Brazil		N.I.	Reduced parasitemia enhanced the survival of the animals, and did not induce any hepatic, muscular lesion, or renal toxicity (25–300 mg/Kg)	[135]

N.I. = none identified.

4.3. Leishmaniasis

Leishmaniasis is a neglected disease group, occasioned by 20 species of protozoan parasites belonging to the genus *Leishmania* and spread by female sand flies of the genus Phlebotomus or Lutzomyia. Present in nearly 100 countries and endemic in Asia, Africa, the Americas, and the Mediterranean region, more than 12 million people, about 25,000 deaths, and 1 million new cases are reported annually; according to the WHO, it is a Category I (emerging or uncontrolled) disease [136]. In humans, four clinical forms of this disease can develop: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), or post-Kala-azar dermal leishmaniasis (PKDL). For more than six decades, pentavalent antimonials (SbV) were the first-line drugs against leishmaniasis; however, the toxicity and resistance of the parasites are the main limitation of these drugs [137]. Other treatments such as pentamidine, paromomycin, or amphotericin B has been employed, but its high costs and side effects make it difficult to use [137,138]. Therefore, alternatives are urgently required that complement and help with the adequate treatment of leishmaniasis. Propolis has been studied as an alternative to various protozoa, including parasites of the genus *Leishmania*. The main investigations on the leishmanicidal effect of propolis have mainly been conducted in vitro, focusing on determining the effect of propolis on the mortality of this parasite, e.g., the brown, green, and red propolis from Brazil and that from Portugal, which showed significant growth inhibition of *L. braziliensis* Vianna, *L. infantum*, and *L. amazonensis* promastigotes, and decreased the number of internalized amastigotes in infected murine macrophages [84,139–141]. Some of the countries with the highest incidence of Leishmaniasis are in the Middle East; the administration of propolis as an antileishmania agent has also been reported in this region. The composition of propolis from three regions of Turkey (Adana, Hatay, and Bursa) was analyzed, and differences were found in the type of compounds and in their quantities; the main component of Adana propolis was found to be cembrene, that of Hatay was chrysin, and Bursa's was cinnamyl cinnamate. All three registered a good antileishmanial activity against *L. tropica* or *L. infantum*, but the propolis from Bursa was the most effective [142,143]. The effects of propolis on Leishmaniasis have been studied more: as mentioned earlier, various in vitro studies have shown the benefits of this apiculture product. Fortunately, in several of these studies, the chemical compounds present in each propolis were identified. Several of these pure compounds have been tried individually against different species of *Leishmania*, and these compounds are likely related to the antileishmanial effect of propolis [144–146]. Although these studies are limited, since they only involved in vitro tests, they provide support for the use and application of propolis in animal models.

Many have evaluated the effect of propolis from Latin American countries on various species of the genus *Leishmania* and have identified a large part of the chemical composition of these propolis. For example, 20 propolis from Cuba presented in vitro antimicrobial properties; the major effect was found on *L. infantum*. The results demonstrated an association between the biological effect and compounds identified. The propolis that contain acetyl

triterpenes as amyrin, lupeol, and cycloartenol as the main constituents are the best options for future studies [120]. Similarly, three propolis obtained from distinct areas in Ecuador (Quito, Guayaquil, and Cotacach) avoided *L. amazonensis* growth, highlighting the activity of sample rich in flavonoids as naringenin, sakuranetin, eupatolitin, and rhamnazin [147]. Brazil is one of the countries with the most studies of the biological and chemical properties of its propolis. Brazilian propolis (Ribeirao Petro and Minas Gerais) were proven on *Leishmania* species associated with different clinical forms of leishmaniasis, and the chemical composition was determined. Propolis from Minas Gerais showed great antileishmanial effect on *L. amazonensis*, *L. braziliensis*, *L. chagasi*, and *Leishmania major*, with the last species being the most susceptible. Ribeirao Petro propolis was only evaluated against *L. amazonensis*; it recorded a dose-dependent activity against promastigotes, also the number of parasites decreased inside macrophages. Although a leishmanicidal effect on *L. amazonensis* was reported in the two studies, the effects were not the same, because the extracts from Minas Gerais and Ribeirao Petro had different chemical compositions: the main compounds of the first were diethyl 2-methylsuccinate, cinnamic acid, pentanedioc acid, and hydrocinnamic acid; for the second, they were artepillin C, 4,5-dicaffeoylquinic acid, *p*-coumaric acid, and drupanin [148,149]. Brazilian propolis also showed strong in vivo effects: in an experimental infection model with *L. braziliensis* using BALB/c mice treated previously with propolis, it reduced growth and promoted morphologic alterations on promastigotes and also favored the TNF-α levels in supernatants from liver cells and peritoneal exudate [150]. Green propolis decreased more than 75% in lesion development caused by L. brazilienzis, while the glucantime treatment showed a 57.7% decrease (Table 13) [151].

Table 13. Antileishmanial activity of various propolis and its chemical composition.

Propolis Origin	Parasitic Species	Identified Compounds	Activity	Ref.
Brazil (brown, green, and red propolis) and Portugal	*L. braziliensis*, *L. infantum*, and *L. amazonensis*	N.I.	All inhibited the growth of promastigotes of distinct parasitic strains and effectively reduced number of internalized amastigotes in infected murine macrophages (36–250 mg/mL)	[84,139–141]
Turkey (Three regions: Adana, Hatay, and Bursa)	*L. tropica* and *L. infantum*	Adana: cembrene; Hatay: chrysin; Bursa: cinnamyl cinnamate	All propolis (50–1000 μg/mL) showed good antileishmanial activity, but that of Bursa was the most effective	[142,143]
Cuba (20 different samples)	*L. infantum*	Amyrin, lupeol, and cycloartenol	All samples of propolis (3.2–22.2 μg/mL) presented antiprotozoal properties	[120]
Ecuador (three different samples)	*L. amazonensis*	Naringenin, sakuranetin, eupatolitin, and rhamnazin	All inhibited *L. amazonensis* growth, but the sample rich in flavonoids was the most effective (12.5–200 mg/mL)	[147]

Table 13. *Conts.*

Propolis Origin	Parasitic Species	Identified Compounds	Activity	Ref.
Brazil (two different samples)	*L. amazonensis*, *L. braziliensis*, *L. chagasi*, and *L. major*	Minas Gerais: diethyl 2-methylsuccinate, cinnamic acid, pentanedioc acid, and hydrocinnamic acid; Ribeirao Petro: artepillin C, 4,5-dicaffeoylquinic acid, *p*-coumaric acid, and drupanin	Both showed great antileishmanial activity. Ribeirao Petro propolis exhibited a dose-dependent effect against promastigotes of *L. amazonensis* and controlled the parasite burden inside infected macrophages (2.8–229.3 μg/mL)	[148,149]
Brazil	*L. braziliensis*-infected mice	N.I.	Reduced growth by promoting morphologic alterations in promastigotes; in supernatants from liver cells and peritoneal exudate of mice pretreated with propolis and infected, increased TNF-α production was seen (5–100 μg/mL)	[150]
Brazil (green propolis)		N.I.	Decreases lesion development caused by *L. braziliensis* more than 75%, while the glucantime treatment showed a 57.7% decrease (10–250 μg/mL)	[151]

When used in combination with nitric oxide (NO) during infection with *L. amazonensis* at the lesion site, the levels of NO, healing, collagen synthesis, the function of macrophages and fibroblasts were favored, in addition to decreased parasitized cells, pro-inflammatory factors, and tissue damage [152]. Propolis from Brazil was also used in combination with first line antileishmaniasis medications. Green propolis was administered in combination with liposomal meglumine antimoniate, decreasing the parasitic burden in the liver without damaging or altering the functions of the kidney, liver, spleen, and heart (Table 14) [153]. The two works mentioned in this section are noteworthy as they used propolis as a complementary or combination treatment with another substance. This type of study demonstrates the path that can be followed in the examination of propolis and its bioactive compounds, since propolis is not intended to replace existing treatments but to supplement them with new alternatives [154,155]. Finally, clinical trials are still needed to demonstrate the effectiveness of propolis in humans.

Table 14. Activity of Brazilian propolis on different *Leishmania* infection models.

Propolis Origin	Substances	Infection Model	Activity	Ref.
Brazil	Nitric oxide	*L. braziliensis*-infected mice	The combination of propolis (5 mg/kg) with NO favored the healing, collagen synthesis, the function of macrophages and fibroblasts, reduced expression of proinflammatory and tissue damage markers	[152]
Brazil (green propolis)	Liposomal meglumine antimoniate	*L. infantum*-infected mice	Reduced the parasitic burden in the liver, without damaging kidney, liver, spleen, and heart (500 mg/kg)	[153]

4.4. Giardiasis

Giardiasis is a parasitic intestinal disease, the etiological agent of which is *Giardia duodenalis*, also known as *G. intestinalis* or *G. lamblia*. This parasite is transmitted mainly by consuming water or food that contains Giardia cysts. Symptoms of infection are usually diarrhea, nausea, epigastric pain, and weight loss. Giardiasis annually affects about 200 million people worldwide. Since 2004, it is listed by the WHO as a neglected disease by the World Health Organization. The prevalence of *Giardia* infection is higher in developing countries [156,157]. Several remedies of traditional medicine have been administered as a complement to treat this disease [158], among which is propolis.

There are reports of the in vitro effect of three samples from Sonoran Desert propolis in Mexico (Caborca, Pueblo de Alamos, and Ures) and some of its bioactive compounds. The Ures propolis presented a remarkable activity on *G. lamblia* in a dose-dependent manner, as well as one of its components (CAPE), which registered the highest antigiardia effect [159]. Similarly, propolis from Brazil showed efficacy in eliminating trophozoites of *G. lamblia*. The effect on the proteolytic activity of excretory/secretory products (ESPs) from trophozoites treated with propolis was studied; however, no significant differences were found between hydrolysis patterns and inhibition on the protease activity of propolis-treated and untreated trophozoites [160,161]. Propolis from Egypt was reported to be effective in an in vivo model of giardiasis with immunodeficient mice. The propolis produced a diminution in intensity of infection, as well an augment in the IFN-γ serum level and in the $CD4^+$:$CD8^+$ T cell ratio. Combination propolis and metronidazole presented a great effect in reducing the number of parasites than that produced by each drug alone. Futhermore, this combination induced an immunological regulation, mainly in T lymphocytes, which favor intestinal homeostasis and histological integrity (Table 15) [162]. While several drugs against this parasitosis are available, its incidence continues to be higher in developing countries [163]. In these regions, it is common for people to use traditional or alternative medicine to address health problems [164]. For this reason, it is necessary to determine the effectiveness of these treatments against this parasite. The works included in this review on this disease demonstrate the in vitro and in vivo effect of propolis and its effect on the immunological response against *G. lamblia*. Further research is important to support the utility of propolis as an alternative against Giardiasis; the work conducted to date and the information available are limited.

In this work, we addressed the properties of propolis in the defense against these parasitic diseases; in most cases, propolis showed interesting and promising antiprotozoal activities [165]. However, several challenges remain, such as the wide variety in the components of propolis in each geographical area, isolating the components responsible for the activities, and describing their mechanisms of action and synergy. It is also necessary to promote and conduct research using animal models, since very few studies have been published. It should be noted that the implementation of clinical studies is necessary to support the antiparasitic activity of propolis, as well as conducting research focused on the combined treatment of propolis with different drugs used to treat parasitic infections in

humans, and to find more effective complementary treatments in order to be able to reduce the dose and toxicity of the drugs currently implemented.

Table 15. Effect of different propolis on *G. lamblia* in in vitro and in vivo models.

Propolis Origin	Parasitic Species	Activity	Ref.
Mexico (Sonoran Desert, region Ures)	*G. lamblia*	Inhibitory activity against *G. lamblia* in a dose-dependent manner (63.8 μg/mL)	[159]
Brazil		Effectively eliminated trophozoites of *G. lamblia* (125 μg/mL)	[160,161]
Egypt	*G. lamblia*-infected immunodeficient mice	Reduces infection, enhanced IFN-γ serum level and CD4+:CD8+ T cell ratio. Co-administration of propolis and metronidazole had remarkable activity in controlling the parasite number. Favors intestinal homeostasis and the histological integrity (NS)	[162]

NS: not specified.

4.5. Helminths

Humans are exposed to a remarkable number of parasites, including protozoans (over 70 species), helminths (about 300 species), and arthropod parasites. There are two major phyla of helminths: the nematodes (also known as roundworms) and the Platyhelminthes (Trematoda and Cestoda) [166,167]. According to the WHO, more than 2 billion cases of intestinal worms were registered in 2018, mainly affecting disadvantaged communities [168]. However, this number could be higher, since many of these diseases are not reported [169]. Another relevant aspect regarding helminths is that they not only parasitize humans, but also affect many domestic animals and the livestock industry, resulting in large economic losses [168]. In addition, in the absence of therapeutic options in developing regions, this population resorts to the use of traditional remedies or alternative medicine to treat these parasites.

The anthelmintic activity of some propolis from Egypt on adult flukes of *Fasciola gigantica* was reported. Alteration of the architecture was found as lifting base of the spines and large blisters in the apical cone, several of which seemed to have burst, generating injuries. The inhibitory activity on the viability and hatchability of immature *F. gigantica* eggs was also found, showing the highest inhibitory effect compared with other treatments. The chemical composition of these propolis was determined. Compounds such as diprenyl-dihydrocoumaric acids, coumarate esters, ferulate esters, hydroxy acetophenones, furanon derivative, furofuran lignans, benzofuran lignans, and valeric acids derivatives could be related to anthelmintic activity [170,171]. Other propolis from Egypt was evaluated against *Schistosoma mansoni* in mice: propolis alone or in combination with praziquantel were administrated. Propolis administration did not eliminate the worms of infected mice but significantly reduced the hepatic granuloma number, hepatic, splenic, and plasma myeloperoxidase (MPO) activity, as well the liver and thymus NO levels, and also regulation of plasma antioxidant proteins evidenced by decrease in malondialdehyde (MDA) and normalization of glutathione (GSH) [172]. The antihelmintic activity of propolis from Turkey on *Echinococcus granulosus* was reported: 1 μg/mL of propolis killed all the protoscoleces in the in vivo part of the study, without causing side effects when administered intraperitoneally. However, the mechanism of action and chemical composition were not reported [173]. There are also reports of the anthelmintic effect of the essential oil of Brazilian red propolis, larvae of *Toxocara cati*, were incubated during 48 h with the essential oil, and then later inoculated in mice. The authors informed 100% effectivity to disable the infective capacity of the larvae [174]. Five propolis from distinct parts of Libya were studied, and they presented moderate activity against *Trichinella spiralis*. The components of the propolis was analyzed, and fourteen compounds were identified, of which cycloartanol, mangiferolic

acid, agathadiol, isocupressic acid, and isoagatholal were highlighted (Table 16) [175]. These compounds may play interesting roles in the effects of propolis on helminths in each of the studies reviewed. As each propolis has a complex and changing chemical composition, it is a priority to determine whether the antihelmintic activity is due to a specific compound or a synergism phenomenon to identify new pharmacological alternatives [176]. Since the results in these works show the favorable effects of propolis against various helminths, these propolis could be tested against parasites such as *Tenia*, *Enterobius*, and *Ascaris*, which have high incidence in several countries and are a public health problem [177,178].

Table 16. Anthelmintic activity of propolis and its chemical composition.

Propolis Origin	Parasitic Species	Identified Compounds	Activity	Ref.
Egypt	*F. gigantica*	Diprenyl-dihydrocoumaric acids, coumarate esters, ferulate esters, hydroxy acetophenones, furanon derivative, furofuran lignans, benzofuran lignans, and valeric acids derivatives	Alteration in the architecture, inhibitory activity on the viability and hatchability of immature helminths (10–800 μg/mL)	[170,171]
	S. mansoni-infected mice	N.I.	Propolis administration (500 mg/kg) not eliminated the worms of infected mice, but significantly reduced the hepatic granuloma number, hepatic, splenic and plasma MPO activity, as well the liver and thymus NO levels; also, regulation of plasma antioxidant proteins evidenced by decrease in MDA and normalization of GSH	[172]
Turkey	*E. granulosus*	N.I.	Propolis (1 μg/mL) killed all the protoscoleces	[173]
Brazil (essential oil of red propolis)	*T. cati*	N.I.	Have 100% larvicidal effect after treatment (1 μg/mL) of 48 h and can suppress the ability of the treated *T. cati* larvae to infect the mice	[174]
Libya (five different samples)	*T. spiralis*	Cycloartanol, mangiferolic acid, agathadiol, isocupressic acid, isoagatholal	All propolis samples (4.7–59.3 μg/mL) showed moderate activity	[175]

N.I., none identified; MPO, myeloperoxidase; MDA, malondialdehyde; GSH, glutathione.

Helminth parasites mainly continue to be a public health problem in countries with disadvantaged and low-resource communities. Currently available anthelmintic drugs include the benzimidazoles (albendazole and mebendazole), pyrantel pamoate, and ivermectin [179]. Although these drugs are usually well-tolerated and efficient for the treatment of helminth parasites, they are limited in number, and the susceptibility among helminth species has been shown to vary greatly in different populations [180]. Another concern is the emergence of resistance, which have mainly been observed in veterinary medicine over the past decade [181]. Traditional and alternative medicine offers a wide repertoire of compounds that could complement the treatment of these diseases. Research in animal models must be increased, and clinical trials are needed to confirm the safe utilization of propolis in these diseases.

5. Antiviral Activity of Propolis

Viruses need the host cells' biosynthetic machinery to replicate [182,183]. Viral infections are responsible for some diseases in humans and cause serious public health problems in populations worldwide [184]. Therefore, recent research on new antiviral medications is increasing due to the development of resistance to antiviral drugs [185]. As such, the study of natural products that present antiviral activity, such as propolis and some of its identified compounds, is vital [186,187].

Two propolis from Czech Republic (aqueous and ethanolic extracts) were studied, and both showed great antiviral effect on herpes simplex virus type 2 (HSV-2). Both propolis decreased the infection and exhibited a concentration- and time-dependent antiviral effect. Additionally, the two propolis showed a high antiviral effect when viruses were pretreated prior to infection; thus, both propolis could be used to treat recurrent herpetic infection topically [188].

In other research, the antiviral effect of different propolis from several geographic regions, such as the United States, Brazil, and China, was against human immunodeficiency virus type 1 (HIV-1). All propolis inhibited viral expression in $CD4^+$ lymphocytes and microglial cell in a concentration-dependent manner. In another study, propolis from the United States suppressed cell fusion HIV-1 in cultures of $CD4^+$ lymphocytes, suggesting that the possible mechanism of propolis' antiviral property in $CD4^+$ lymphocytes is produced in part by inhibition viral entry into cells [189].

The antiviral activity of four Brazilian propolis on influenza virus was examined, and all propolis presented anti-influenza virus effect in vitro. In this same study, the four propolis were studied in a murine influenza virus infection model (propolis was orally administered three times daily for seven days), and only one propolis sample effectively prolonged the lifetime of infected mice. The authors concluded that the Brazilian propolis possessed antiviral effect and amelioration influenza symptoms in mice [190]. In another study, three different extracts of Brazilian propolis were administered orally three times daily for six days to cutaneous herpes simplex virus type 1 (HSV-1)-infected mice to study their effect on HSV-1 infection. The three propolis presented anti-HSV-1 activity and favored immunological effect on intradermal HSV-1 infection in mice (Table 17) [191]. At present, diseases caused by viruses are a priority in any health system due to the severity of their symptomatology, their high infective capacity, and mortality. Viral diseases can affect the economy of a country or the globe [192,193]. The previously mentioned propolis studies are notable and highly relevant, since they reported antiviral properties capable of inhibiting viral replication, cell fusion in cultures of $CD4^+$ lymphocytes, and stimulation of immunological activity [189]. However, these works lack a chemical analysis, which limits the explanation of some possible action mechanisms related to the secondary metabolites present in each propolis.

Some flavonoids and phenolic acids, also described in propolis, presented antiviral activity [187]. One antiviral study showed that Canadian propolis had a pronounced viricidal effect against HSV-1 and HSV-2 and interfered with virus adsorption. Different compounds were identified in this propolis. The interaction with propolis indicates damage to the HSV and suggests that propolis could damage protein components of envelopes essential for adsorption and penetration of the virus into the cells (Table 18) [194].

Table 17. Antiviral activity of different samples of propolis.

Propolis Origin	Viral Species	Activity	Ref.
Czech Republic (aqueous and ethanolic extract)	HSV-2	Both propolis (0.0005–0.005%) decreased the infection and exhibited a concentration- and time-dependent antiviral effect; high antiviral effect when viruses were pretreated prior to infection	[188]
United States, Brazil, and China	HIV-1.	Three propolis inhibited viral expression in CD4+ lymphocytes and microglial cell in a concentration-dependent manner; propolis from United States suppressed cell fusion in cultures of CD4+ lymphocytes (0.8–66.6 μg/mL)	[189]
Brazil (13 different samples)	Influenza virus.	Four samples had anti-influenza virus effect in vitro (10–149.2 μg/mL)	[190]
	Influenza virus-infected mice.	Only one propolis sample (10 mg/mL) effectively prolonged the lifetimes of infected mice; anti-influenza effectiveness of propolis in mice was dose-dependent	
Brazil (13 different samples)	HSV-1-infected mice	The three propolis had direct anti-HSV-1 effects, stimulated immunological effect on intradermal HSV-1 infection in mice (0.4, 2, and 10 mg/mL)	[191]

Table 18. Anti-HSV-1 and HSV-2 activity of several propolis and its chemical composition.

Propolis Origin	Parasitic Species	Identified Compounds	Activity	Ref.
Canada	HSV-1 and HSV-2	Benzoic acid, cinnamic acid, vanillic acid, *p*-coumaric acid, ferulic acid, caffeic acid, palmitic acid, oleic acid, pinocembrin, pinobanksin, chrysin, galangin, isosakuranetin, alpinone, kaempferol, pinostrobin chalcone, and pinocembrin chalcone	Presents a pronounced viricidal effect and interfered with virus adsorption (0.1 mg/mL)	[194]
Turkey (south)		Gallic acid, (±)-catechin, caffeic acid, syringic acid, epigallocatechin, *p*-coumaric acid, trans-ferulic acid, trans-isoferulic acid, myricetin, trans-cinnamic acid, benzoic acid, daidzein, luteolin, pinobanksin, (±)-naringenin, apigenin, kaempferol, chrysin, pinocembrin, galangin, and CAPE	Suppressed the replication of HSV-1 and HSV-2; inhibited HSV-1 replication following 24 h of incubation and effect on HSV-2 at 48 h following incubation; decreased the number of viral copies; activity similar to that of acyclovir; a synergism of the propolis and acyclovir combined on HSV-1 and HSV-2 replication compared with acyclovir alone (25–3200 μg/mL)	[185]
Czech Republic (aqueous and ethanolic propolis)	HSV-1	Caffeic acid, *p*-coumaric acid, benzoic acid, galangin, pinocembrin, and chrysin	Both samples presented high anti-HSV-1 effect in cells pretreated prior to viral infection; galangin and chrysin were the most bioactive compounds; however, the propolis had higher antiherpetic effects than single isolated constituents (1%)	[187]

Similarly, the antiviral effect of aqueous and ethanolic extract of Czech Republic and some compounds identified in propolis against HSV-1 in cell culture was analyzed. Both samples presented high anti-HSV-1 effect in cells effect in prior to viral infection; of the compounds tested, galangin and chrysin were the most active components. However, the propolis with various compounds presented higher antiviral activities than the isolated constituents alone. The authors concluded that the antiherpetic activity of propolis is due to a combination of several components; therefore, the propolis from Czech Republic was found to be more effective on herpes infection than the individual compounds [187].

In another study, the replication of HSV-1 and HSV-2 was inhibited with the propolis from the south of Turkey. Propolis started to suppress HSV-1 replication after 24 h of incubation and effect on HSV-2 started at 48 h after incubation. This activity of propolis on HSV-1 and HSV-2 was checked by a lower in the number of viral copies. They found that propolis showed activity similar to that of acyclovir, since both started to suppress HSV-1 replication following 24 h of incubation. They also found a synergistic effect of combined propolis and acyclovir on HSV-1 and HSV-2 replication compared with acyclovir alone. Some compounds in the propolis were identified. The propolis from the south of Turkey was found to present relevant antiherpetic activities in comparation with acyclovir; particularly, the synergism generated by the antiherpetic effect of propolis and acyclovir in combination has a stronger activity on HSV-1 and HSV-2 than acyclovir alone. The authors mentioned that the possible mechanism of synergism between acyclovir and propolis may be attributed to some of the components of propolis [185]. As described in each work, most of the molecules identified in each propolis are compounds of phenolic origin. Some of these have been reported as having the ability to stimulate antiviral responses in in vitro and in vivo models, promoting the production of interferons as well as the activation of cytotoxic T lymphocytes and natural killer cells [195–199]. This supports the use of propolis as a source of new molecules with antiviral effects and an alternative to complement the treatment of these diseases.

Although little literature about the antiviral activity of propolis in clinical studies exists, in 2019, Jautová et al. reported that one lip cream with propolis extract from Central Europe produced a better effect than acyclovir to treat patients with herpes labialis in the vesicular phase, confirming the clinical efficacy of lip cream composed of European propolis in the early and late start of treatment during an episode of herpes labialis [200]. Similarly, a clinical study reported the contribution of propolis extract from Central Europe as a constituent in a lotion for complementary treatment of Herpes zoster. A total of 33 patients with a diagnosis of Herpes zoster applied a treatment with a propolis-based lotion for 28 days as a complementary treatment to oral antiviral treatment with acyclovir. The healing of lesions was improved and faster with the propolis treatment; approximately 50% of propolis-treated patients had no injuries on day 14 and the growth of new vesicles was inhibited, clinically confirming the antiviral effects of European propolis and demonstrating the properties of complementary therapy on the systemic antiviral treatment of Herpes zoster (Table 19) [201].

Table 19. Antiherpetic activity of Central European propolis in patient studies.

Propolis Origin	Model	Activity	Ref.
Central Europe (lip cream with propolis)	Patients with herpes labialis	Lip cream with propolis (0.5%) produced a better effect than acyclovir in the treatment of patients with herpes labialis in the vesicular phase	[200]
Central Europe (constituent of a lotion)	Herpes zoster in patients	Improvement in pain and healing of lesions were better and quicker with treatment of the propolis lotion (0.5%); approximately 50% of propolis-treated patients had no injuries on day 14 and the formation of new vesicles was suppressed	[201]

The current situation related to COVID, which has compromised all health systems, makes it necessary to search for therapies that prevent or mitigate the complications of this disease. Natural products such as propolis are an interesting option in the search of complete therapies. Some recent research mentions the potential benefits of using propolis against this disease. These studies are based on previously reported activities against other viruses and on in silico models that allow predictions of activities against this virus. These studies focus mainly on reported bioactive compounds in the different propolis; they include antiviral activities that could be applied against SARS-CoV-2 or immunomodulatory effects that would reduce the symptoms of the disease. One of the clearest examples is quercetin, one of the most abundant and consumed flavonoids in the diet. Quercetin has been shown to inhibit the replication cycle of the virus, since it reduces the functioning of the main protease (Mpro) and S protein of SARS-CoV-2. CAPE, one of the main components of many propolis, is able to inhibit the transmembrane protease serine 2 (TMPRSS2), angiotensin-converting enzyme-related carboxypeptidase (ACE-II), and Mpro; these molecules are crucial for access and replication viral of SARS-CoV-2 in cells. Another interesting compound is the rutin that reduced the function of S protein, ACE-II, and others non-structural proteins of SARS-CoV-2. These flavonoids are also able to regulate JAK/STAT-mediated signaling and the production of ROS, NO, pro- and anti-inflammatory cytokines, avoiding a cytokine storm. They even reduce the risk of comorbidities that complicate the betterment of patients with COVID-19 [202–204]. Propolis and its bioactive compounds open new means for future works that describe in detail their effects on SARS-Cov-2 and are able to be applied as a complementary therapy in clinical studies.

Considering all the research mentioned above, the search for new strategies for the control and complementary treatments of infections caused by viruses has become a global public health priority. However, more in vitro and clinical studies with propolis are needed to elucidate its mechanisms of action and identify the molecules responsible for the antiviral effects of this natural product.

6. Conclusions

We collect the main studies of the effect of propolis on pathogens related to infectious diseases of medical relevance. The reports of the efficacy of the different propolis are encouraging: this bee product showed effectiveness on bacteria, fungi, protozoa, helminths, and viruses. Propolis presents a great spectrum of components that could be used to treat characteristic affections of distinct diseases. Not all propolis present the same activities; depending on the flora of the geographical area, each propolis has a different chemical composition with unique biological activities, making propolis a promising source of discovering molecules, which can be used in different clinical situations. Propolis offers potential for research into the treatment of infectious diseases that lack adequate therapies due to the resistance of pathogens to drugs, either isolating active components to be studied alone or combined with different current drugs. Despite the in vitro and in vivo evidence suggesting that propolis can be a reliable alternative to existing drugs, the effect of propolis must be investigated in the clinic to improve tour comprehension of the mechanisms of action of the different propolis, attain the synergism of their compounds, and generate a standardized and safe consumption protocol.

Another relevant aspect is that clinical tests with propolis, bee products, or other natural products are scarce but necessary. From products used in traditional medicine, modern medicine has obtained compounds such as taxol, valproic acid, polycarpine, ephedrine, digoxin, and acetylsalicylic acid, just to name a few. The therapeutic uses and applications of natural products and their derivatives are promising in the search for new treatments, so clinical studies against diseases caused by microbes resistant to drugs or treated with toxic agents should be a priority in future clinical research.

Finally, a new perspective to consider in future research is to investigate the presence and function of microRNAs (miRNAs) in propolis. Recent studies have proposed that the miRNAs present in honey from plants visited by bees during their collection could play a determining role in the development of larvae. The finding of these molecules

could be surprising related to the beneficial effects on the health of consumers of this bee product. The identification of miRNAs in propolis would be crucial to understanding and explaining many of its biological and medicinal activities, and these activities are currently attributed mainly to compounds such as flavonoids and terpenes. miRNAs in bee products can be the subject of various investigations, and their clinical applications could generate new treatments based on nutritional supplements with various specific benefits for health [205,206].

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Effects on Steroid 5-Alpha Reductase Gene Expression of Thai Rice Bran Extracts and Molecular Dynamics Study on SRD5A2

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Simple Summary: Dihydrotestosterone (DHT), the most potent androgen hormone, is an important aetiologic factor of androgenetic alopecia (AGA), or hair loss. Steroid 5-alpha reductases (SRD5As) increase DHT production in the scalp hair follicles, resulting in hair thinning and hair loss. Even though synthetic SRD5A inhibitors (finasteride and dutasteride) are effective in treating AGA, they cause adverse effects. This has led to an increased interest in alternative treatments from natural sources. The value of Thai rice bran has increased because several of its components may have use in AGA treatment. This study aimed to compare the suppression of the expression of *SRD5A* genes (type 1–3) exerted by several Thai rice bran extracts and investigate the interactional mechanism of their components towards SRD5A type 2. Tubtim Chumphae rice bran (TRB) had the highest sum of overall bioactive compounds. Among all extracts, the expression of *SRD5A* genes was suppressed by TRB as well as finasteride. In silico simulation showed that α-tocopherol had the greatest interaction with SRD5A type 2. Our findings identified α-tocopherol as the key bioactive in TRB; it could be developed as an anti-hair loss product.

Abstract: Steroid 5-alpha reductases (SRD5As) are responsible for the conversion of testosterone to dihydrotestosterone, a potent androgen, which is the aetiologic factor of androgenetic alopecia. This study aimed to compare the *SRD5A* gene expression suppression activity exerted by Thai rice bran extracts and their components and investigate the interactional mechanism between bioactive compounds and SRD5A2 using molecular dynamics (MD) simulation. Bran of *Oryza sativa* cv. Tubtim Chumphae (TRB), Yamuechaebia Morchor (YRB), Riceberry (RRB), and Malinil Surin (MRB), all rice milling by-products, was solvent-extracted. The ethanolic extract of TRB had the highest sum of overall bioactive compounds (γ-oryzanol; α-, β-, and γ-tocopherol; phenolics; and flavonoids). Among all extracts, TRB greatly downregulated the expression of *SRD5A1*, *SRD5A2*, and *SRD5A3*; there were no significant differences between TRB and finasteride regarding *SRD5A* suppression. The linear relationship and principal component analysis supported that the α-tocopherol content was correlated with the *SRD5A* suppression exerted by TRB. Furthermore, MD simulation demonstrated

that α-tocopherol had the highest binding affinity towards SRD5A2 by interacting with residues Phe118 and Trp201. Our findings indicate that α-tocopherol effectively downregulates the expression of *SRD5A* genes and inhibits SRD5A2 activity, actions that are comparable to standard finasteride. TRB, a source of α-tocopherol, could be developed as an anti-hair loss product.

Keywords: 5α-reductase; androgenetic alopecia; rice bran; tocopherol; SRD5A2; molecular dynamics; RD69; Tubtim chumphae

1. Introduction

Androgenetic alopecia (AGA), characterised by the progressive replacement of terminal hair into small vellus hair, is generally known as a hereditary androgen-dependent disorder [1]. Although AGA is not a serious threat to health, it may impact the social and psychological well-being of an individual [2]. Different tissues, including hair follicles, require an optimal androgen concentration. High androgen levels can have deleterious effects on health [3]. Overexpression and intensive activity of steroid 5-alpha reductase (SRD5A) in scalp follicles have been shown to be involved in AGA development [4].

SRD5A comprises five members, SRD5A1, SRD5A2, SRD5A3, and the little characterised glycoprotein synaptic 2 (GSPN2) and GSPN2-like [5]. SRD5As are dihydronicotinamide adenine dinucleotide phosphate (NADPH)-dependent [6] and play a significant role in steroidogenesis by catalysing 4-ene-3-keto steroids into more active 5α-reduced derivatives, including the reduction of testosterone (T) to dihydrotestosterone (DHT) [7]. SRD5A1, SRD5A2, and SRD5A3 are encoded by separate genes: *SRD5A1*, *SRD5A2*, and *SRD5A3*, respectively. While these isozymes share sequence homology and show similar substrate preferences, they vary in biochemical properties, sensitivity to SRD5A inhibitors, physiological functions, and also tissue distribution [8]. DHT has a 5-fold higher affinity for the androgen receptor and a 10-fold greater potency for provoking androgen-sensitive genes compared with its precursor [9]. These androgen-sensitive gene products, transforming growth factor beta 1, interleukin 6, and dickkopf 1, have been identified as androgen-inducible negative mediators for AGA development [10].

SRD5A genes are expressed differently in androgen-responsive tissues, which include the adrenal glands, the testis, the placenta, and the skin. When translated, the proteins are located mainly in the endoplasmic reticulum membrane [11]. Beyond sexual functions, they have also been implicated in and influence the diverse biological activities of the skin [12]. Both SRD5A1 and SRD5A2 are well-characterised enzymes involved in AGA; they are expressed principally in skin and annexes, including hair follicles, sweat glands, and sebaceous glands [4]. The expression levels of *SRD5A1* and *SRD5A2* are higher in the frontal hair follicles in both men and women with AGA, a pattern that indicates they play a major role in AGA [13]. The level of *SRD5A2* expression is higher in dermal papilla cells (DPCs) from an AGA scalp than in DPC from other sites [1]. In contrast, *SRD5A3* is involved in protein *N*-glycosylation and shows slight or no potential to catalyse steroid substrates [8,14]. Interestingly, a previous study identified that the ratio of the total amounts of DHT to T is significantly depleted in *SRD5A3*-knockdown cells [15]. Moreover, tissue distribution analysis demonstrated that the expression level of *SRD5A3* is higher in peripheral tissues, including the skin, compared with *SRD5A1* and *SRD5A2* [16]. *SRD5A3* is overexpressed in prostate cancer [17], and several studies have proposed an association between prostate cancer and AGA [18,19]. A subsequent study also reported that a higher expression level of *SRD5A3* in plucked hair derived from AGA, suggesting its possible role in the pathogenesis of AGA [20].

To date, oral finasteride and dutasteride, SRD5A competitive inhibitors, have been approved to treat AGA. Finasteride specifically inhibits SRD5A2 as well as SRD5A3 but restrains less effectively SRD5A1. On the other hand, dutasteride exhibits great inhibitory effects against the three distinct types of SRD5A [5,16]. Despite their promising efficacy,

these drugs are associated with several side effects, especially erectile dysfunction and loss of libido [21]. In recent years, several herbal extracts and their bioactive constituents have been implemented as an alternative treatment to promote hair growth or prevent hair loss [22].

Rice (*Oryza Sativa* Linn.) is used as a staple food by more than half of the globe [23]. Thailand is recognised as the biggest rice exporter and the fifth-biggest producer in the world [24]. Rice bran is an important by-product that is largely generated during the milling process [25]. In addition, it is a rich source of biologically active compounds, including γ-oryzanol, phytic acid, vitamin E isoforms (α-, β-, and γ-tocopherol), unsaturated fatty acids (such as oleic acid, linoleic acid, and γ-linolenic acid), and phenolic compounds (such as ferulic acid, gallic acid, and caffeic acid) [26]. The geographical origin and genetic diversity among rice varieties also influence the types, appearances, and bioactive content in rice bran [25].

Rice bran and its biomolecules have been shown to possess the potential for application as a treatment for hair loss and androgen-dependent disorders such as benign prostatic hyperplasia, hirsutism, and hypertrichosis [22]. Researchers suggest that specific aliphatic unsaturated fatty acids, especially oleic acid, linoleic acid, and γ-linolenic acid, inhibit the activity of SRD5A in androgen-responsive tissue [27]. In addition, various extracts from *Serenoa repensits*, *Thujae occidentalis*, *Cucurbita pepo*, and *Panax ginseng* inhibit SRD5A activity; these findings suggest that free fatty acids contribute to SRD5A inhibition [22]. Rice bran supercritical CO_2 extracts and linoleic acid have been reported to suppress the messenger RNA (mRNA) expression of *SRD5A1* in cell lines [28]. Several studies have focused on the effect of plant extracts or their bioactive constituents, especially unsaturated fatty acids, on the inhibition of SRD5A activities, but only a few have focused on the gene expression levels [22].

Previous studies have investigated the inhibitory effects of rice bran extracts and their constituents on SRD5A1 and SRD5A2, but the effect on SRD5A3 is still unknown. Furthermore, the effect of other major components (vitamin E isoforms, phytic acid, and phenolic compounds) of rice bran extracts on the expression of *SRD5A* genes has not been thoroughly determined [29]. SRD5A2 is an important causative factor of AGA, and the aforementioned study was limited to SRD5A2. Moreover, the atomic-level mechanism between SRD5A2 and the bioactive compounds of rice bran extracts remains unclear. Molecular dynamics (MD) simulation can help to understand the binding mode of ligands towards SRD5A2, screen potential ligands, and accelerate candidate identification instead of the experimental method. With regards to these rationales, it is necessary to understand the biochemical actions of rice bran extracts and their bioactive compounds before converting this agricultural waste to a high-value-added anti-hair loss product. Hence, this study aimed to compare the *SRD5A* suppression exerted by four Thai rice bran varieties and their bioactive compounds and also to investigate the interactional mechanism at an atomic level of bioactive compounds in rice bran towards SRD5A2.

2. Materials and Methods

2.1. Reagents and Chemicals

Sulphorhodamine B (SRB); the Folin–Ciocalteu reagent; (−)-epigallocatechin gallate (EGCG); ferulic acid, phytic acid, gallic acid, oleic acid, linoleic acid, and γ-linolenic acid; γ-oryzanol; and α-, β-, and γ-tocopherol were obtained from Sigma Chemical (St. Louis, MO, USA). Finasteride and dutasteride were obtained from Wuhan W&Z Biotech (Wuhan, China). Agarose gel, Tris base, and 50X Tris/acetic acid/EDTA (TAE) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Foetal bovine serum (FBS; cat no. 16000044) and Roswell Park Memorial Institute medium (RPMI-1640; cat no. 31800022) were obtained from Gibco Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Penicillin/streptomycin solution (100X) was purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Ethanol, dimethyl sulphoxide (DMSO), acetic

acid, trichloroacetic acid, and other chemical substances were obtained from RCI Labscan (Bangkok, Thailand). All other chemicals were of analytical grade.

2.2. Plant Material and Extraction

Rice bran of *Oryza sativa* Linn. cv. Tubtim Chumphae (RD69; TRB), Riceberry (RRB), and Mali Nil Surin (SRNC05053-6-2; MRB) was provided by Phrao Green Valley Co., Ltd. (Chiang Mai, Thailand). Rice bran of Yamuechaebia Morchor (YMCB 3 CMU; YRB) was obtained from Lanna Rice Research Center, Chiang Mai University, Thailand. Herbarium voucher specimens of TRB (PNPRDU63021), YRB (PNPRDU63022), RRB (PNPRDU63023), and MRB (PNPRDU63024) were deposited in the Pharmaceutical and Natural Products Research and Development Unit, Faculty of Pharmacy, Chiang Mai University. Rice bran (2 kg) was macerated in 95% (*v*/*v*) ethanol (ratio of solid/solvent: 1:3) for 24 h. The extract solutions were filtered through Whatman filter paper no. 4 and then no. 1 and concentrated by a vacuum evaporator (Hei-VAP value, Heidolph, Schwabach, Germany) at 50 °C. All extracts were stored in a sealed vial in the dark at 4 °C before further analysis.

2.3. Determination of Bioactive Compounds

2.3.1. γ-Oryzanol and Tocopherols

The high-performance liquid chromatography (HPLC) conditions were adapted from a previous study [30]. The analytical system consisted of an Agilent 1220 Infinity DAD LC module (Agilent Technology, Palo Alto, CA, USA) and a fluorescent detector (Agilent 1260 FLD Spectra, Agilent Technology). The separation was performed on an Ultra C-18 column (250 mm × 4.6 mm, 5 µm particle size; Restek, Bellefonte, PA, USA). Briefly, 10 mg of the sample was diluted with 1 mL of isopropanol, mixed and then filtered through a 0.45 µm syringe filter into a 1.5 mL vial. Twenty microliter aliquots were injected. A mixture of acetonitrile/methanol/isopropanol in different ratios served as the mobile phase (solvent A: 50:40:10, *v*/*v*/*v*; solvent B: 30:65:5, *v*/*v*/*v*). The following procedure was used for the separation of both γ-oryzanol and tocopherols: isocratic elution with phase A for 5 min, followed by a 10 min linear gradient from phase A to 100% phase B, and a final 5 min isocratic elution with phase B. The column temperature was 25 °C with the flow at 1 mL/min. γ-Oryzanol was detected by a UV–VIS spectrophotometric detector at 325 nm. Tocopherols were detected by using a fluorescence detector with excitation and emission wavelengths at 290 and 330 nm, respectively. OpenLAB software (Agilent Technology) was used to acquire and process the data. The standard compounds were γ-oryzanol and the mixture of α-, β-, and γ-tocopherol.

2.3.2. Total Phenolic Content

The total phenolic content (TPC) of all samples was determined using the Folin–Ciocalteu colourimetric method, as described in a previous study [31]. The calibration curve was plotted using the absorbance of standard gallic acid against its concentration in the range between 0.2 and 0.0016 mg/mL. The TPC was calculated according to the standard curve equation of gallic acid ($y = 13.463x + 0.0406$, $R^2 = 0.9991$) and is expressed as mg gallic acid equivalents per 100 g dried sample (mg GAE/100 g). All the samples were prepared in triplicate.

2.3.3. Total Flavonoid Content

An aluminium chloride colourimetric assay was used to estimate the total flavonoid content (TFC) of all extracts following the method described by Zeng et al. [32]. The calibration curve was created using different concentrations of EGCG (0.01–0.32 mg/mL) and its absorbance was measured at 515 nm. The standard curve equation of EGCG was y = 0.3587x + 0.0041 (R^2 = 0.9993). The results are represented as mg EGCG equivalents per 100 g dried sample (mg EGCGE/100 g). All the samples were prepared in triplicate.

2.4. Cell Culture

DU-145 human prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). DU-145 was grown in RPMI-1640 containing 10% FBS and 1% antibiotics (100 µg/mL of streptomycin and 100 unit/mL of penicillin). DU-145 cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. The cells in passages 3–6 were used for all experiments.

2.5. Cell Viability Assay

The samples were tested to determine the non-cytotoxic concentration and cell viability of DU-145 cells by using SRB assay, as previously described [33]. Briefly, cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and incubated overnight for cell attachment in a 5% CO_2 atmosphere at 37 °C. Cells were then exposed to five serial concentrations (0.0001–1 mg/mL) of the ethanolic rice bran extracts (TRB, YRB, RRB, and MRB), their bioactive compounds (ferulic acid, phytic acid, gallic acid, oleic acid, linoleic acid, and γ-linolenic acid; γ-oryzanol; and α-, β-, and, γ-tocopherol), and standard controls (dutasteride and finasteride). Control cells were treated with 10% (*v*/*v*) DMSO in incomplete RPMI-1640, whereas incomplete RPMI-1640 served as a blank. After 24 h treatment, the adherent cells were fixed with 50% (*w*/*v*) trichloroacetic acid for 30 min, and cells were washed with water and then air-dried. Cells were stained with 0.04% (*w*/*v*) SRB for 30 min. The unbound dye was removed by washing with 1% (*v*/*v*) acetic acid. The bound stain was solubilised with 10 mM Tris base, and absorbance was detected at 515 nm using a 96-well plate reader (EZ Read 400 Flexi, Biochrom, Cambridge, UK). The experiments were performed in triplicate. The highest non-toxic concentration that gave more than 80% cell viability was selected for further studies. The percentage of cell viability was calculated by Equation (1), where Abs denotes absorbance:

$$\text{Cell viability } (\%) = \left(\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}} \right) \times 100. \quad (1)$$

2.6. RNA Extraction and Semiquantitative RT-PCR Analysis

2.6.1. RNA Extraction

Total RNA was extracted from DU-145 cells treated with 0.10 mg/mL of the ethanolic rice bran extracts (TRB, YRB, RRB, and MRB), 0.01 mg/mL of bioactive compounds, 0.10 mg/mL of standard controls (finasteride and dutasteride), or untreated cells using the NucleoSpin® RNA isolation kit (cat no. 740955.50; Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The concentration of isolated RNA was quantified using a Qubit 4 fluorometer (Invitrogen, Carlsbad, USA) and Qubit™ RNA HS Assay Kit (Invitrogen). The total RNA solution was kept at −20 °C until use.

2.6.2. Semi-Quantitative RT-PCR

Complementary DNA (cDNA) was synthesised by using the RT-PCR Quick Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Briefly, the 20 µL reaction mixture contained 1 µg of total RNA, 10 µL of the RT-PCR Quick Master Mix, 1 µL of manganese acetate, 1.2 µL of oligo dT primers (Integrated DNA Technologies, Coralville, IA, USA), and nuclease-free water. The transcript levels of the genes of interest (*SRD5A1*, *SRD5A2*, and *SRD5A3*) and the reference gene (glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)) were measured in triplicate. The sequences of primers are listed in Table 1. The cycle consisted of denaturation at 94 °C for 30 s, annealing at 50–55 °C for 30 s, and extension at 72 °C for 1 min; there were 40 amplification cycles.

The RT-PCR products were analysed by electrophoresis on 1% (*w*/*v*) agarose gels in a chamber with 1X TAE buffer at 100 V for 60 min. The gel was imaged with a Gel Doc™ EZ System (Version 3.0; Bio-Rad) to obtain images of the bands. Image Lab™ software (Bio-Rad) was used to analyse quantitatively the intensity of the bands. The expression of target genes was normalised by the GAPDH expression value and is expressed as the

relative expression (*RE*) value. The percentage of SRD5A suppression was calculated according to Equation (2):

$$SRD5A \text{ suppression } (\%) = \left(\frac{RE_{control} - RE_{sample}}{RE_{control}} \right) \times 100 \quad (2)$$

Table 1. The sequences of the primers used for RT-PCR.

Primer	NCBI Reference Sequence	Forward Primer	Reverse Primer
SRD5A1	001047.4	AGCCATTGTGCAGTGTATGC	AGCCTCCCCTTGGTATTTTG
SRD5A2	000348.4	TGAATACCCTGATGGGTGG	CAAGCCACCTTGTGGAATC
SRD5A3	024592.5	TCCTTCTTTGCCCAAACATC	CTGATGCTCTCCCTTTACGC
GAPDH	001289745.3	GGAAGGTGAAGGTCGGAGTC	CTCAGCCTTGACGGTGCCATG

2.7. Computational Method Details

2.7.1. Protein and Ligand Preparation

The structure of human SRD5A2 comprises seven transmembrane domains and six loops that construct a cavity inside SRD5A2 (Figure 1). A carboxyl-terminal side (C-terminal side) faces the cytosol, whereas an amino-terminal side (N-terminal) faces the endoplasmic reticulum lumen. The space between transmembrane (TM)1 and TM4 is the entry port that opens for the ligand to enter the binding site. Loop 1 (L1) has been suggested to be a gate domain that controls the NADPH/$NADP^+$ exchange from the cytosol. A previous study reported that SRD5A2 catalyses the hydride transfer from NADPH to finasteride, resulting in the formation of a stable intermediate adduct, namely NADP–dihydrofinasteride (NADP–DHF), via a covalent bond [34]. Consequently, SRD5A2 is inhibited irreversibly. The key residues E57 (Glu57), R114 (Arg114), and F118 (Phe118) inside the SRD5A2 pocket have been suggested to interact with the intermediate adduct of finasteride and steroid substrate [34].

The crystal structure of human SRD5A2 in complex with NADPH and finasteride was retrieved from the Protein Data Bank (PDB) with the PDB ID 7BW1 [34]. SRD5A2 was prepared in Discovery Studio version 2.5 software. In the SRD5A2 binding pocket, finasteride is already fused with $NADP^+$. The finasteride structure was removed by breaking the chemical bond, while the reduced form of $NADP^+$ (NADPH) only remained in the substrate-binding cavity. SRD5A2 with NADPH in the binding pocket (Figure 1) is the state before ligand entry and formation of the intermediate adduct. This model was used to evaluate and compare the binding mode of ligands. The structure of finasteride retrieved from the crystal structure was used as a positive control.

The three-dimensional structure of nine bioactive compounds (ferulic acid, phytic acid, oleic acid, linoleic acid, γ-linolenic acid, γ-oryzanol, and α-, β-, and γ-tocopherol), which can be purified from rice bran extracts and dutasteride, another positive control, were retrieved from the PubChem compound database and optimised with the Gaussian 09 program (Gaussian Inc., Wallingford, CT, USA) using the B3LYP model with a 6-31G (d, p) basis set [35]. Figure 2 illustrates the chemical structures of selected ligands.

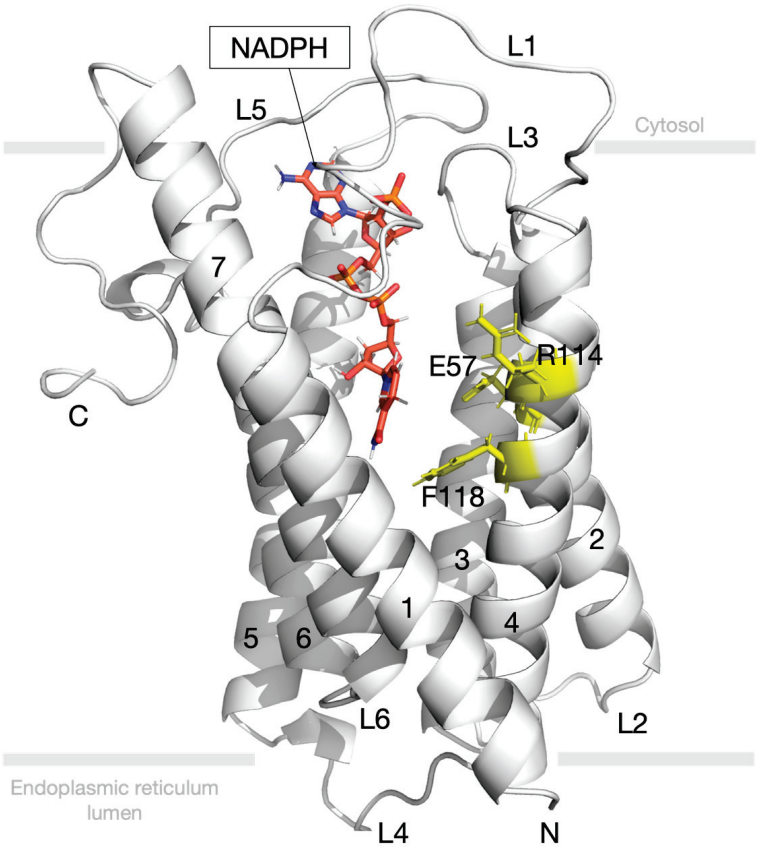

Figure 1. Structure of steroid human 5-alpha reductase 2 (SRD5A2) comprises seven transmembranes (7 TMs) and six loops. Dihydronicotinamide adenine dinucleotide phosphate (NADPH) (orange) is located inside the binding site. Crucial residues involving ligand interaction are coloured in yellow.

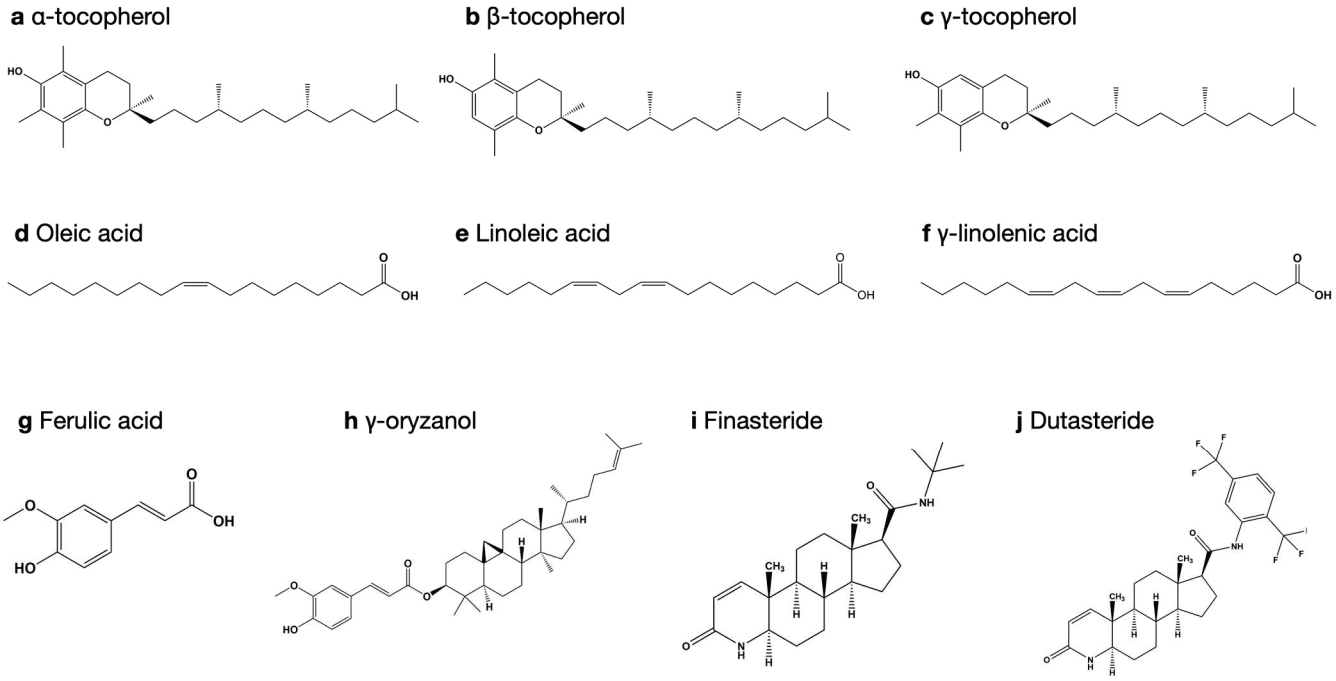

Figure 2. Chemical structure of selected ligands. (**a**) α-tocopherol, (**b**) β-tocopherol, (**c**) γ-tocopherol, (**d**) oleic acid, (**e**) linoleic acid, (**f**) γ-linolenic acid, (**g**) ferulic acid, (**h**) γ-oryzanol, (**i**) finasteride, and (**j**) dutasteride.

2.7.2. Molecular Docking Study for 5-Alpha Reductase 2

Nine compounds and two positive controls were docked into the binding site of SRD5A2 using AutoDock 4.2.6 and AutoDockTools, the graphical user interface [36]. In all dockings, a grid box was generated with a centre over the native finasteride position. Grid box dimensions were set to 34, 30, and 30 in x, y, and z dimensions, respectively. The grid spacing was kept at 0.375 Å. One hundred conformations were generated by Lamarckian genetic algorithm searches with an initial population size of 300 random positions and conformation. Each run had two stop criteria: a maximum of 2,500,000 energy evaluations and a maximum of 27,000 generations. The reference root-mean-square deviation (RMSD) was kept as the default, with a mutation rate of 0.02 and a crossover rate of 0.8. The RMSD tolerance of 2 Å was kept for clustering of docked poses and ranked according to their binding energy. The docked conformation with the lowest binding energy of the most populated cluster was selected.

2.7.3. Molecular Dynamics Simulation

The best-docked complexes of native finasteride (positive control); α-, β-, and γ-tocopherol; the best top-three compounds; and SRD5A2 were selected for the MD simulation using the AMBER 14 program. The Amber ff14SB force field was used for the conformational analysis of protein systems [37,38]. For the non-standard unit in AMBER, antechamber was used to determine the GAFF atom type for structures of the four ligands and NADPH, and the restrained electrostatic potential (RESP) charges were employed for these ligands [39]. The systems were neutralised with 14 chloride counterions and centred in a 10 Å truncated octahedral box of pre-equilibrated TIP3P water molecules using the tLEaP program [40]. All MD simulations were carried out with the GPU-capable PMEMD.CUDA in AMBER14. The solvated structures were first energy-minimised to remove possible steric stress using the steepest descent (SD) method and the conjugate gradient techniques (CONJ) with a different part of the system [41]. They were then heated gradually from 0 to 310 K for 500 ps and equilibrated at 310 K at 1 atm pressure to obtain a stable density for 1000 ps. The unconstrained production simulations were run in an NPT ensemble at 310 K and 1 atm for 50 ns. The Langevin thermostat was used to maintain the temperature of the system [42], the SHAKE algorithm was used to constrain all of the chemical bond lengths involving hydrogen atoms [43], and the time step was set at 2 fs for all MD simulations.

2.7.4. Trajectory Analysis

Visual molecular dynamics (VMD) [44] and PyMOL [45] were used to visualise and analyse MD trajectories. The structural analysis of the conformational ensemble was performed by evaluating the RMSD with the CPPTRAJ module implemented in AMBER 14 [46]. The binding free energy and decomposed binding free energy of SRD5A2/ligand complexes were evaluated by the molecular mechanics–generalised Born surface area (*MM/GBSA*) protocol using the MMPBSA.py module, as embedded in AMBER 14 [47,48]. The snapshots were extracted from 50 ns of MD trajectories for the analysis of the binding free energy; all water molecules and chloride counterions were removed prior to calculations. In *MM/GBSA*, binding free energy (Δ*Gbind*) was estimated through Equation (3) [49]:

$$\begin{aligned} \Delta G_{bind} &= G_{complex} - G_{protein} - G_{ligand} \\ &= \Delta H + \Delta G_{solvation} + T\Delta S \\ &= \Delta E_{MM} + \Delta G_{GB} + \Delta G_{SA} - T\Delta S \\ &= \Delta E_{vdw} + \Delta E_{ele} + \Delta G_{GB} + \Delta G_{SA} + T\Delta S \end{aligned} \quad (3)$$

where Δ*EMM* is the gas-phase interaction energy between protein and ligand, containing van der Waals interaction energy (Δ*Evdw*) and electrostatic energy (Δ*Eele*); Δ*GGB* and Δ*G* denote the polar and nonpolar desolvation free energy, respectively; and -*T*Δ*S* indicates the conformational entropy contribution at temperature *T*, where *T* is the absolute temperature

and *S* the entropy of the molecule. Here, the generalised Born (*GB*) approximation model was used to estimate the polar desolvation term (ΔGGB) [50,51], while the solvent-accessible surface area (*SASA*) model with the LCPO model used to estimate the nonpolar desolvation term (ΔGSA): $\Delta GSA = 0.0072 \times \Delta SASA$ [52].

2.8. Statistical Analysis

Data are expressed as the mean of three independent experiments ± standard deviation. Principal component analysis (PCA) of bioactive contents and *SRD5A* suppression was performed using a free trial version of XLSTAT (Addinsoft, New York, NY, USA). Linear correlation between variables is expressed by Pearson's correlation coefficient (*r*). Analysis of variance (ANOVA) followed by post hoc analysis (Tukey's test) was used to compare means and evaluate statistical differences. The null hypothesis was rejected at the calculated probability value of less than 5%. Both linear correlation and ANOVA were carried out in SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Extraction Yield and Bioactive Compounds

The extraction yields, relative to 100 g of dried material, were 7.49% ± 0.89% (TRB), 5.68% ± 0.27% (YRB), 4.54% ± 0.92% (MRB), and 3.08% ± 1.67% (RRB); TRB had the highest yield among the four varieties of rice bran extracts. The physical appearances of all extracts were viscous semisolid and greasy with different colours due to pigment deposition in the pericarp or the bran of the rice kernel [26,53], specifically dark reddish-brown (TRB and YRB), black-purple (MRB), and dark purple (RRB).

The active constituents of rice extracts are presented in Table 2. Tocopherols are the major group of natural antioxidants in rice bran and have a similar chemical structure based on a 6-chromanol amphiphilic ring with one to three methyl groups and a phytyl tail with three chiral centres, resulting in α-, β-, γ-, and δ-tocopherol [26]. The highest content of α-tocopherol was found in TRB (20.76 ± 0.13 mg/kg extract), followed by YRB (12.52 ± 0.01 mg/kg extract), RRB (11.95 ± 0.04 mg/kg extract), and MRB (7.61 ± 0.01 mg/kg extract). Pigmented rice bran contains about 9.67–116.60 mg/kg of α-tocopherol, which is higher than that in non-pigmented rice bran [54]. β-Tocopherol is present at only minor concentrations in rice bran extracts [55], and the pair of β- and γ-tocopherol isomers is not completely separated using reversephase HPLC [30]. Hence, the pair of β- and γ-tocopherol was quantified and interpreted jointly as (β+γ)-tocopherol. The content of (β+γ)-tocopherol in YRB (50.98 ± 0.02 mg/kg extract) was much higher than in the other rice bran extracts, while δ-tocopherol was not detected in the extracts. These results are in agreement with several studies, where α- and β-tocopherol are predominantly present in rice bran and rice whole grain [55,56].

Rice bran is a major source of γ-oryzanol, a mixture of steryl ferulates, and speculated to be the primary constituent of rice bran oil [26]. The content of γ-oryzanol was enriched in TRB (8600.45 ± 0.13 mg/kg extract) and RRB (9174.01 ± 0.09 mg/kg extract). It has been reported that the mean value of γ-oryzanol in non-pigmented rice bran is approximately 3067.10 mg/kg [54]. Overall, pigmented TRB and RRB contained a high content of γ-oryzanol, and black-purple rice (MRB) had the lowest amount of tocopherols and γ-oryzanol. It has been suggested that environmental factors as well as the origin and genotype of rice varieties influence the composition of steryl ferulates, resulting in the variation of the γ-oryzanol profiles in black-purple rice varieties [57].

Phenolic and flavonoid contents were measured by colourimetric methods. Highest levels of TPC and TFC were found in red rice bran (YRB): 254.97 ± 5.20 mg GAE/100 g dried sample and 880.16 ± 22.86 mg EGCGE/100 g dried sample, respectively. Phenolic acids can be classified as free, conjugated, and bound. The free form is suggested to be easily extracted from rice bran [53]. Phenolic compounds, including phenolic acids and flavonoids, are mainly present in pigmented rice and act as metal ion chelators, free radical scavengers, and reducing agents [53,58]. The foremost phenolic acids found in bran

include ferulic acid (56–77% of total phenolic acids), followed by *p*-coumaric acid, sinapic acid, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, and syringic acid [46]. A previous study reported that Thai red rice bran contains a higher TPC than black and white rice bran extracts, resulting in better antioxidant activity [59]. The main flavonoids in non-pigmented rice varieties are flavones, whereas proanthocyanidins and anthocyanins are primarily found in pigmented rice varieties [54,60]. Anthocyanins and proanthocyanidins are responsible for purple-to-blue pigmentation and red pigmentation, respectively [26,61]. Moreover, a study reported that red and purple bran rice has greater TPC and TFC than light-coloured bran rice and other cereals due to higher concentrations of proanthocyanidins and anthocyanins, respectively [62].

Table 2. Bioactive compounds of four types of rice bran extracts.

Sample	α-Tocopherol mg/kg Extract	(β+γ)-Tocopherol mg/kg Extract	γ-Oryzanol mg/kg Extract	TPC mg GAE/100 g	TFC mg EGCGE/100 g
TRB	20.76 ± 0.13	23.32 ± 0.01	8600.45 ± 0.13	180.44 ± 6.42	569.01 ± 90.42
YRB	12.52 ± 0.01	50.98 ± 0.02	3773.17 ± 0.01	254.97 ± 5.20	880.16 ± 22.86
MRB	7.61 ± 0.01	3.51 ± 0.01	ND	125.67 ± 0.49	545.30 ± 48.33
RRB	11.95 ± 0.04	16.97 ± 0.01	9174.01 ± 0.09	56.47 ± 2.82	441.49 ± 12.39

Rice bran extracts of Tubtim Chumphae (TRB), Yamuechaebia Morchor (YRB), Mali Nil Surin (MRB), and Riceberry (RRB). Total phenolic content (TPC) presented as mg gallic acid equivalents per 100 g of dried sample (mg GAE/100 g). Total flavonoid content (TFC) expressed as mg (−)-epigallocatechin gallate equivalents per 100 g of dried sample (mg EGCGE/100 g). Not detected (ND).

Several studies have reported the use of topical α-tocopherol in cosmetic applications and treatments of cutaneous diseases such as wounds, sunburn, atopic dermatitis, and hair loss [63–65]. A recent study reported that α-tocopherol-loaded hydrogel promoted the healing of the dorsal skin injury in a rat model. Based on the histopathological results, the α-tocopherol-treated group showed epidermal proliferation and the generation of new hair follicles [63]. In addition, α-tocopherol and α-tocopheryl acetate lotions showed the acceleration of the hair growth rate in a rabbit model within two weeks [66]. Nevertheless, whether tocopherols affect an androgen-dependent pathway involving AGA has not been directly observed. Therefore, tocopherols were selected for further study to determine their effect on *SRD5A* isozyme expression and for a molecular docking study. In addition, other bioactive compounds in rice bran extracts, such as ferulic acid [67], phytic acid [68], oleic acid [27,69], linoleic acid [27,69,70], γ-linolenic acid [69,71], and γ-oryzanol [28], which are involved in androgen metabolism pathways, were compared to tocopherols and standard controls (finasteride and dutasteride).

3.2. Effect on the Expression of 5-Alpha Reductase Isoenzymes

The activity of SRD5A in hair follicles was first identified by Takayasu et al. [72]. *SRD5A1* and *SRD5A2* gene expression levels in men were about threefold higher than in women, resulting in the higher prevalence of AGA among men compared with women [4]. The expression of *SRD5A3* has been suggested to be a predisposing factor to AGA development [18–20]. Moreover, the absence of temporal regression and baldness in cases of *SRD5A* deficiency supports the crucial role of *SRD5A* in the pathogenesis of AGA [73]. Downregulation of *SRD5A* gene expression could lead to a reduction in their protein translation in the downstream pathways involving the pathology of AGA. Several studies have indicated that the DU-145 human androgen-insensitive prostate adenocarcinoma cell line expresses the three types of *SRD5A*; these cells have been used in the present study to observe the regulation of *SRD5A* gene expression [16,28,74,75]. With regards to these, the effects of the selected bioactive compounds and rice bran extracts on *SRD5A1*, *SRD5A2*, and *SRD5A3* expression were investigated. The percentage of *SRD5A* suppression and the relative expression of each *SRD5A* gene are given in Supplementary Materials (Table S1 and Figure S1).

The four rice bran extracts significantly decreased the mRNA expression levels of *SRD5A1*, *SRD5A2*, and *SRD5A3* compared with the negative control groups in the follow-

ing order: TRB > RRB > YRB > MMB (Figure 3). Interestingly, treatment with TRB greatly decreased the expression levels of *SRD5A1*, *SRD5A2*, and *SRD5A3* by 35.79% ± 6.94%, 22.26% ± 3.73%, and 21.97% ± 0.01%, respectively (Table S1). Finasteride, a dual inhibitor of SRD5A2 and SRD5A3, suppressed *SRD5A1*, *SRD5A2*, and *SRD5A3* by 54.21% ± 3.05%, 6.48% ± 8.22%, and 7.70% ± 3.58%, respectively. However, there were no significant differences between TRB and finasteride regarding *SRD5A* gene suppression. Dutasteride, a triple inhibitor of SRD5As, downregulated *SRD5A1*, *SRD5A2*, and *SRD5A3* by 43.50% ± 0.01%, 20.84% ± 0.54%, and 45.57% ± 0.03%, respectively (Table S1). Among the selected bioactive compounds, the mRNA levels of *SRD5A1*, *SRD5A2*, and *SRD5A3* were downregulated markedly by γ-tocopherol (0.10 mg/mL), specifically 48.32% ± 4.29%, 42.57% ± 1.91%, and 61.04% ± 9.10%, respectively (Table S1). The overall effect of γ-tocopherol on *SRD5A* gene suppression was significantly greater than dutasteride. However, complete downregulation of *SRD5A* genes may not be the best choice for AGA patients, considering the required androgen balance for normal health and tissue homeostasis [3,4]. In skin, androgens also regulate sebum production and secretion, wound healing, cutaneous barrier formation, and hair growth [4]. Undesirable side effects of long-term use of SRD5A inhibitors on skin changes have been reported, including dry skin, thinning skin, changes in skin texture and tone, and penile and scrotal shrinkage [76,77]. Moreover, excessive suppression may lead to ejaculation problems, erectile dysfunction, sexual anhedonia, decreased sperm count, gynaecomastia, and loss of libido in patients [78].

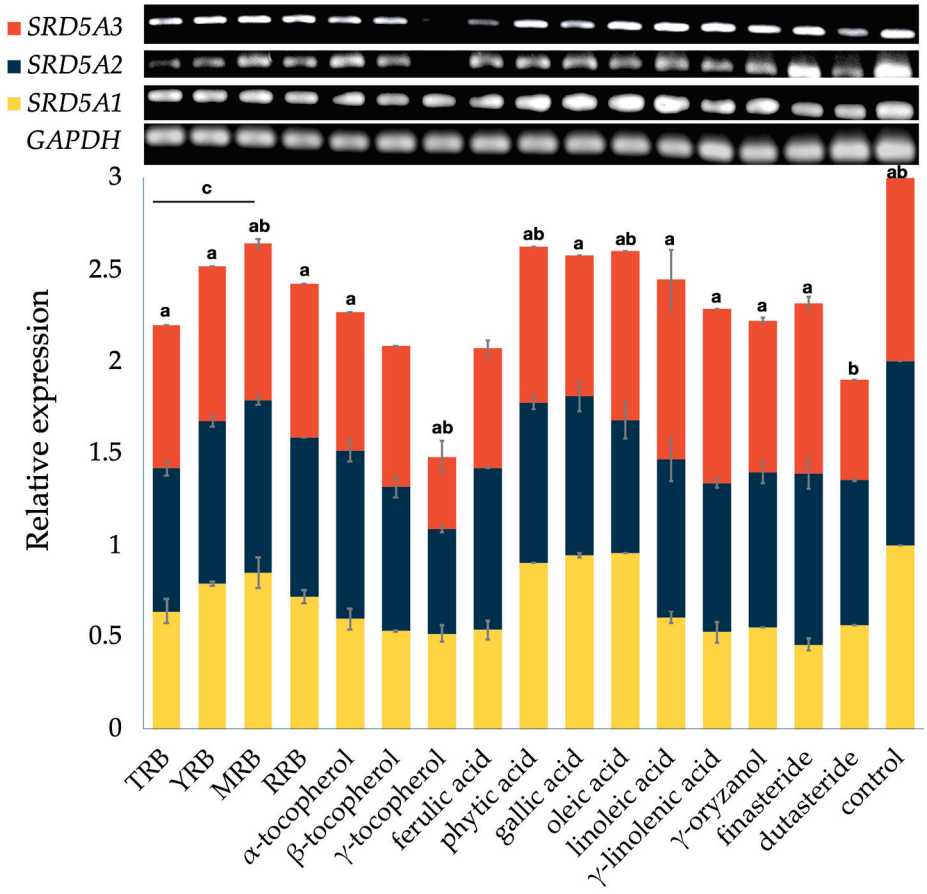

Figure 3. Effects of selected bioactive compounds and rice bran extracts on 5α-reductase isoenzyme (*SRD5A*) expression in DU-145 cells treated with 0.10 mg/mL of ethanolic rice bran extracts (TRB, YRB, RRB, and MRB), 0.01 mg/mL of selected bioactive compounds, and 0.10 mg/mL of standard controls (finasteride and dutasteride). A statistical significance in comparison to dutasteride and finasteride is indicated as a and b ($p < 0.05$), respectively. A significant difference between TRB and other extracts is expressed as c ($p < 0.05$).

The *SRD5A1* mRNA level was significantly lower after treatment with most of the bioactive compounds, including tocopherols, ferulic acid, linoleic acid, γ-linolenic acid, γ-oryzanol, and standard control groups (finasteride and dutasteride), compared with the negative control group (Figure S1a). This result is similar to previous studies [28,79]. The extracts (0.50 mg/mL), including *Nelumbo nucifera*, *Sesamum indicum*, and bran of *O. sativa*, greatly diminished *SRD5A1* expression, suggesting that a high content of linoleic acid is responsible for the activity with a synergist of ferulic acid, vanillic acid, phytic acid, and γ-oryzanol [79]. Nevertheless, in this study, the *SRD5A1* mRNA level slightly decreased in the groups treated with phytic acid, gallic acid, and linoleic acid. The concentration of standard bioactive compounds (0.01 mg/mL) and rice bran extracts (0.10 mg/mL) used in this study was lower than the concentration used in the previous study, suggesting that the *SRD5A* genes may be suppressed in a concentration-dependent manner.

The expression of *SRD5A2* was not remarkably changed with the treatments of YRB, MRB, RRB, and the major compounds (Figure S1b). *SRD5A2* was only downregulated in the cells treated with TRB, β- and γ-tocopherol, oleic acid, γ-linolenic acid, and dutasteride. The *SRD5A2* suppression exerted by the four extracts was in the following order: TRB > RRB > YRB > MMB. TRB suppressed *SRD5A2* by 22.26% ± 3.73%, which was not significantly different compared with standard dutasteride (20.84% ± 0.54%). This suppression might be from the synergistic effect of β- and γ-tocopherol, oleic acid, and γ-linolenic acid in the extract. The *SRD5A3* mRNA level was downregulated significantly in cells treated with all forms of tocopherol, phytic acid, phenolic acids, and γ-oryzanol compared with the control group, but it was not altered in groups treated with unsaturated fatty acids or finasteride (Figure S1c).

The sum of overall bioactive compounds (α-, β- and γ-tocopherol; γ-oryzanol; TPC; and TFC) was ranked in descending order: TRB > RRB > YRB > MMB. In addition, the overall results of *SRD5A* mRNA expression levels from all four rice bran extracts were arranged in the order of decreasing suppression: TRB > RRB > YRB > MMB. Taken together, these bioactive compounds provided potential synergy that enhances the downregulation of *SRD5A* genes.

3.3. Correlation Analysis

3.3.1. Pearson's Correlation

Pearson's correlation coefficients (r) between the potential suppressive effect on *SRD5A* expression and the content of bioactive compounds in four rice bran extracts (α-tocopherol, (β+γ)-tocopherol, γ-oryzanol, TPC, and TFC) were determined to estimate the relationship between variables. The correlation coefficients are classified into four levels: particularly high ($r > 0.9$), high ($0.9 > r > 0.7$), moderate ($0.7 > r > 0.5$), and poor ($r < 0.5$) [80]. There were strong and significant linear relationships between the content of α-tocopherol and the suppressive effect on *SRD5A1* ($r = 0.814$, $p < 0.01$), *SRD5A2* ($r = 0.917$, $p < 0.01$), and *SRD5A3* ($r = 0.943$, $p < 0.01$). In contrast, a previous study reported a significant positive linear correlation between *SRD5A1* suppression and the unsaturated fatty acid content ($r = 1.00$, $p < 0.01$) and the linoleic acid content ($r = 1.00$, $p < 0.01$) [28]. Because the rice bran extracts in the previous study were obtained by supercritical CO_2 extraction, the bioactive compounds in extracts were different from our study, especially the unsaturated fatty acid content [28].

Regarding HPLC results, YRB contained the highest content of (β+γ)-tocopherol among rice bran extracts, followed by TRB, RRB, and MRB. Furthermore, treatment with γ-tocopherol exhibited the greatest suppression of *SRD5A1*, *SRD5A2*, and *SRD5A3*. However, there was not a significant relationship between the content of (β+γ)-tocopherol in rice bran extracts and their suppression of *SRD5A* mRNA levels. Instead, there was a strong relationship between the α-tocopherol content and the suppressive effects on these genes. The most abundant form of tocopherols in pigmented rice bran was α-tocopherol, followed by γ-, β-, and δ-tocopherol [54]. In addition, a study reported that α-tocopherol is the major tocol in two Taiwanese rice varieties [26]. TRB showed the highest α-tocopherol

content, followed by YRB, RRB, and MRB. There was a clear concordance between α-tocopherol and the suppressive effects on *SRD5A* genes. The content of γ-tocopherol in YRB may be lower than the content of α-tocopherol, resulting in the minor effect on gene regulation. These findings indicate that the expression levels of all *SRD5A* genes are greatly downregulated by TRB, suggesting that the higher α-tocopherol content contributes to the more pronounced effect.

3.3.2. Correlation by Principal Component Analysis

PCA was performed to classify the rice bran extracts and cluster the samples. Correlation between rice bran extracts (TRB, YRB, MRB, and RRB) and their biological activity (*SRD5A* suppression) and biological contents (α-tocopherol, (β+γ)-tocopherol, γ-oryzanol, TPC, and TFC) is shown as a PCA biplot in Figure 4. The PCA space distributed 52.64% in PC1 and 34.63% in PC2. The data were separated into four clusters of rice extracts across the PCA space. YRB was separated from other extracts due to the TPC, the TFC, and the content of (β+γ)-tocopherol. TRB showed a high correlation between *SRD5A* gene suppression and the contents of α-tocopherol and γ-oryzanol. RRB was slightly correlated with the content of γ-oryzanol, whereas MRB was not correlated with any variables. Regarding the groups and clustering seen in PCA, the biological activities and biological contents of samples were speculated to be similar. However, there was a strong correlation between the suppression of *SRD5A* genes and the content of α-tocopherol in TRB.

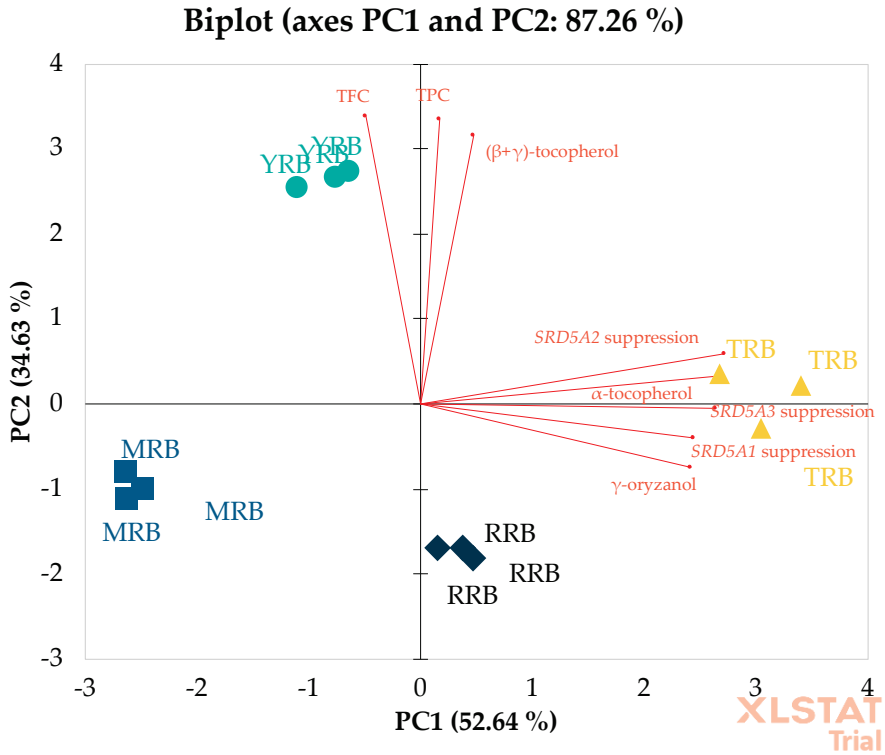

Figure 4. Principal component analysis (PCA) biplot of rice bran extracts and their biological and phytochemical properties. Rice bran extracts of Tubtim Chumphae (TRB), Yamuechaebia Morchor (YRB), Mali Nil Surin (MRB), and Riceberry (RRB). Total phenolic content (TPC). Total flavonoid content (TFC).

3.4. Molecular Docking Study for 5-Alpha Reductase 2

Both *SRD5A1* and *SRD5A2* are expressed and active in scalp hair follicles. Specialised fibroblasts in hair follicles or DPC, known as androgenic targets, induce surrounding epidermal cells to form hair follicles and regulate the hair growth cycle [81]. *SRD5A2* is expressed mainly in DPC obtained from scalp hairs, and its activity is 14-fold higher

than in the remaining hair follicles [4]. In addition, the absence of AGA in males with congenital SRD5A2 deficiency provides strong evidence that SRD5A2 activity is the most importance factor in AGA development [82]. Even though it is more favourable for SRD5A1 to catalyse androstenedione as a substrate to generate 5α-androstenedione, SRD5A2 has been implicated mostly in the reduction of T to DHT [6,83,84]. Furthermore, finasteride, a selective SRD5A2 inhibitor, is a Food and Drug Administration (FDA)-approved drug to treat AGA and has proven favourable efficacy in increasing hair density and hair diameter [85]. Thus, SRD5A2 was selected to perform the further molecular docking with selected bioactive compounds of rice bran extracts.

Molecular docking was performed to predict the potential target/ligand interaction. The binding free energy, which indicates the ligand-binding possibilities with target protein SRD5A2, was calculated. The predicted binding free energies of finasteride, dutasteride, and nine bioactive compounds are ranked and presented in Table 3. The ranking is based on the binding affinity of the SRD5A2/ligand complex; the lowest binding energy (highest negative value) is projected to specify the best-possible interaction. The results indicated that the binding energy of finasteride (−10.13 kcal/mol) was lower than that of dutasteride (−8.75 kcal/mol). These results are in agreement with a previous study that indicated finasteride more specifically inhibits SRD5A2 (IC_{50} = 14.3 ± 2.7 nM) compared with dutasteride (IC_{50} = 57.0 ± 6.8 nM) [5,16]. Several studies have shown that both synthetic and plant-derived unsaturated fatty acids inhibit SRD5A and, consequently, block the conversion of T to DHT [27,33,86,87]. Ferulic acid, γ-oryzanol, and phytic acid appeared to have unfavourable interactions. Similarly to a previous study, there was no correlation between SRD5A inhibitory activity and the TPC [88]. Strikingly, tocopherols displayed a higher affinity towards SRD5A2 compared with dutasteride and other active constituents. The binding free energies of tocopherol are in ascending order: β-tocopherol (−9.83 kcal/mol), γ-tocopherol (−9.53 kcal/mol), and α-tocopherol (−9.47 kcal/mol). Among all considered compounds, finasteride and tocopherols were subjected to MD simulation.

Table 3. Binding free energy of finasteride, dutasteride, and nine bioactive compounds with 5α-reductase 2.

Compounds	AutoDock Binding Free Energy, ΔG (kcal/mol)
Native finasteride	−10.13
β-Tocopherol	−9.83
γ-Tocopherol	−9.53
α-Tocopherol	−9.47
Dutasteride	−8.75
γ-Linolenic acid	−7.03
Linoleic acid	−6.62
Oleic acid	−6.49
Ferulic acid	−5.22
γ-Oryzanol	−4.26
Phytic acid	−2.02

3.5. Molecular Dynamics Simulation

3.5.1. Stability of the Molecular Dynamics Trajectories

The structural dynamics at the atomistic level of SRD5A2 upon interacting with all ligands were carried out by MD trajectories over a 50 ns simulation time. The RMSD values of the protein backbone atoms were calculated to determine the stability of each SRD5A2/ligand complex and were plotted relative to the first frame of the original structure (Figure 5a). The RMSD values of the SRD5A2 backbone for all ligands were in the range of 2 Å, establishing their overall stability over the explored timescale [89]. There were large fluctuations of SRD5A2/α-tocopherol and SRD5A2/β-tocopherol at 10–20 ns and 20–30 ns, respectively. All systems reached the same state after 40 ns.

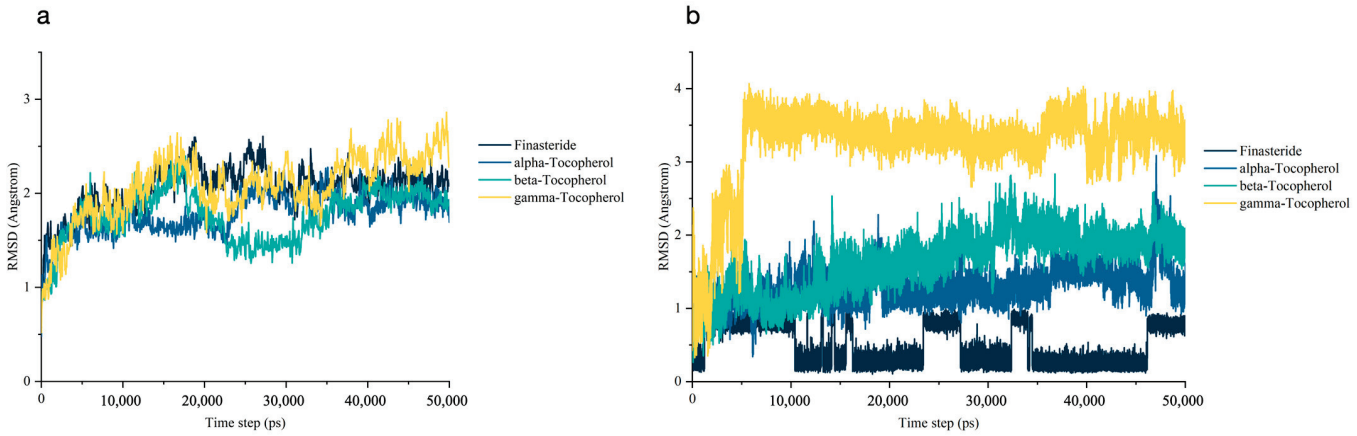

Figure 5. Root-mean-square deviation (RMSD) analysis over 50 ns MD stimulation time: (**a**) RMSD plot of the steroid 5α-reductase 2 (SRD5A2) in complex with each ligand (finasteride and α-, β-, and γ-tocopherol). (**b**) RMSD plot of each ligand.

In the case of the ligand RMSD (Figure 5b), finasteride was stable throughout the 50 ns MD simulation. RMSD plots of α- and β-tocopherol showed almost the same pattern. The RMSD values of three ligands (finasteride and α- and β-tocopherol) were less than ∼2 Å and remained stable to the end of the 50 ns simulation, indicating that these ligands have a stable conformation in the binding site. However, the RMSD of γ-tocopherol increased from the beginning of the MD simulation until approximately 5 ns. Hence, γ-tocopherol dramatically underwent a conformational change at the starting period and then maintained a constant trend until 50 ns. Overall, finasteride and α- and β-tocopherol exhibited a reciprocal stabilisation in the SRD5A2 pocket, contributing to an increase in the binding affinity of the ligands.

3.5.2. Binding Free Energy Analysis

The binding free energy of all ligands towards SRD5A2 was estimated using the snapshots obtained from a 50 ns MD simulation [90]. The different individual energies including van der Waals forces (VDW), electrostatic energy (EEL), nonpolar contribution to the solvation free energy (ESURF), ΔG_{gas}, and ΔG_{solv} are summarised into the total binding free energy, or ΔG_{Total} (Table 4). α-Tocopherol possessed the highest binding affinity against SRD5A2, with ΔG_{Total} of -54.55 ± 3.67 kcal/mol. VDW, a major support of ΔGgas, was more favourable in all ligands compared with electrostatic interaction.

Table 4. Estimated binding free energy of the complex of 5α-reductase 2 with the ligands (finasteride and α-, β-, and γ-tocopherol) using molecular mechanics–generalised Born surface area (MM/GBSA).

Compounds	Binding Free Energy (kcal/mol)						
	VDW	**EEL**	**EGB**	**ESURF**	**ΔG_{gas}**	**ΔG_{solv}**	**ΔG_{Total}**
Finasteride	-53.93 ± 3.23	-18.81 ± 3.41	32.36 ± 3.03	-6.15 ± 0.24	-72.74 ± 4.33	26.20 ± 2.95	-46.54 ± 3.12
α-Tocopherol	-64.97 ± 3.21	-2.26 ± 1.68	20.10 ± 1.80	-7.42 ± 0.39	-67.23 ± 3.68	12.68 ± 1.77	-54.55 ± 3.67
β-Tocopherol	-60.01 ± 4.30	-8.13 ± 2.90	25.88 ± 2.35	-8.15 ± 0.55	-68.14 ± 5.02	17.73 ± 2.10	-50.40 ± 4.33
γ-Tocopherol	-51.24 ± 4.41	-5.71 ± 4.69	23.02 ± 3.76	-6.77 ± 0.47	-56.96 ± 5.54	16.25 ± 3.65	-40.71 ± 3.88

The energy of each compound comprised individual energy terms, including van der Waals forces (VDW), electrostatic energy (EEL), the electrostatic contribution to the solvation free energy (EGB), and nonpolar contribution to the solvation free energy (ESURF). The binding free energy terms are given by ΔG_{gas} (gas-phase free energy) = VDW + EEL, ΔG_{solv} (solvation free energy) = EGB + ESRUF, and ΔG_{Total} (total binding free energy) = ΔG_{gas} + ΔGsolv.

3.5.3. Decomposition of Binding Free Energy

The free energy contribution of each amino acid residue of the SRD5A2 pocket for ligand interaction was elucidated by energy decomposition analysis using MM/GBSA with the configurations based on a 50 ns MD simulation. A residue is considered a

favourable contributor to ligand binding when the total energy decomposition is more negative than −1 kcal/mol [71]. All hotspot residues and NADPH as a donor cofactor are represented in Figure 6. The most crucial residue in all SRD5A2/ligand complexes is Phe118, which contributed favourably to the ligand binding in the following order: α-tocopherol (−9 kcal/mol), β-tocopherol (−6 kcal/mol), finasteride (−4.5 kcal/mol), and γ-tocopherol (−4.2 kcal/mol). It is obvious that finasteride was more likely to interact with NADPH via VDW and *pi*-alkyl interaction. Nevertheless, a covalent bond has been suggested to connect the nicotinamide C-4 atom of NADPH and the C-2 atom at the pyridone ring of finasteride and then create an intermediate adduct between NADPH and finasteride (NADP–dihydrofinasteride) [34,91]. In addition, the key residues in the SRD5A2 binding cavity, such as Ser31, Gly32, Trp53, Glu57, Try91, and Arg94, were prone to form interactions with finasteride. Conversely, tocopherols were mainly involved with the hotspot residues Lue20, Leu11, Arg114, Gly115, Phe219, and Phe233. These findings indicate that finasteride and tocopherols interact with crucial residues in the SRD5A2 pocket at different levels and with distinct binding patterns.

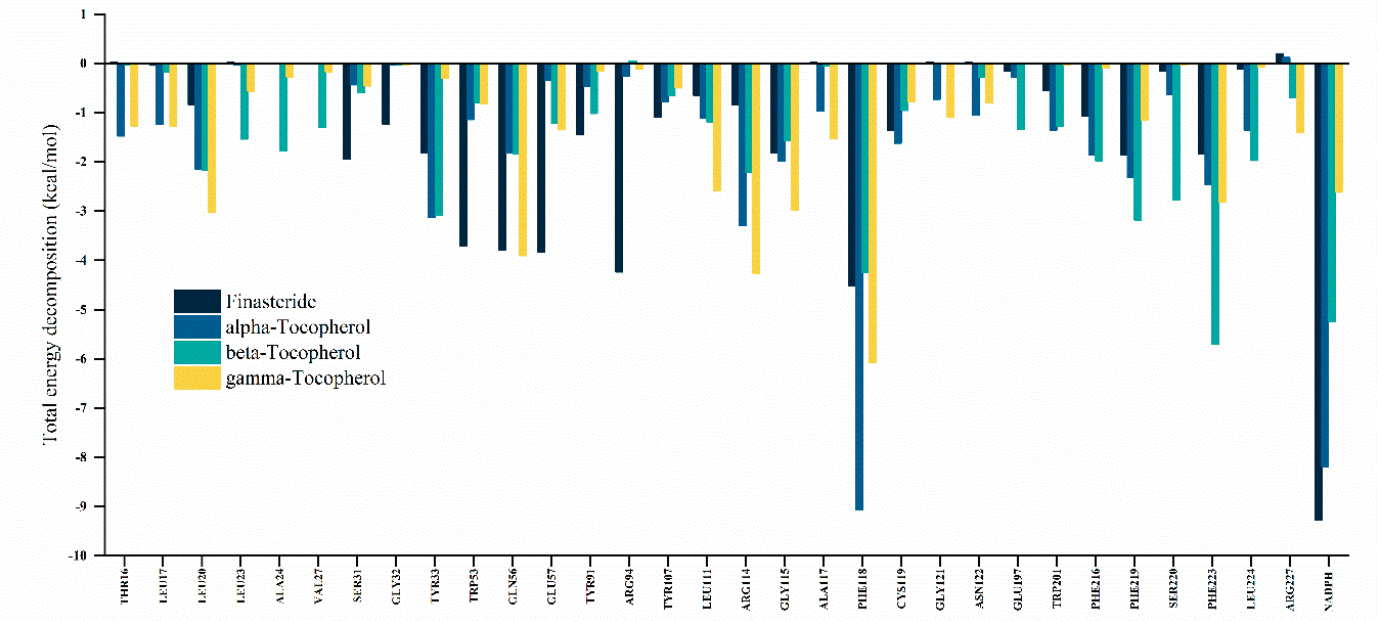

Figure 6. Energy decomposition of amino acid residues in the binding site towards the ligands (finasteride and α-, β-, and γ-tocopherol).

3.6. Post-Molecular Dynamics Simulation Binding Mode Analysis

The dynamic data at atomic spatial resolution revealed that α-tocopherol, which can be found in rice bran extract, is considered a promising SRD5A2 inhibitor. Our preliminary molecular docking study indicated that α-tocopherol, with a binding free energy of −9.47 kcal/mol, had less interaction with SRD5A2 compared with other tocopherols and finasteride. According to the stability of trajectories, all systems of the SRD5A2/ligand complex reached the same state after 40 ns (Figure 5a). The ligand RMSD values of α- and β-tocopherol were quite low, within the acceptable limit of less than 2 Å, whereas the RMSD value of γ-tocopherol was higher (Figure 5b). These data suggest that α- and β-tocopherol undergo a slightly conformational change. However, the binding free energy analysis indicates that α-tocopherol possesses the most favourable interaction by the energy of −54.55 kcal/mol.

Molecular interaction and binding mode conformations of key residues were executed using the final snapshot from the MD simulation of each complex and constructed into 3D and 2D plots (Figure 7). The study demonstrated that tocopherols occupy the SRD5A2

binding pocket similarly to finasteride but display distinct binding patterns. SRD5A2 contains seven transmembrane α-helices with NADPH buried in cytosolic loops [7]. The post-MD binding pose showed that all ligands enter the port and mainly interact with residues in TM2 and TM4.

α-Tocopherol formed different intermolecular interactions between its benzene ring and residues in the binding pocket, including Phe118 (*pi-pi* interaction) and Trp201 (hydrogen bonding). The energy decomposition analysis indicates that NADPH is likely to interact with a chromanol head of α-tocopherol via hydrophobic interactions. The binding environment and the formation of an intermediate adduct between NADPH and α-tocopherol may provide better interaction inside the binding pocket. Moreover, the total energy decomposition of the residue Phe118 is the most negative in the SRD5A2/α–tocopherol complex (−9 kcal/mol). A previous study reported that a substitution mutation at the residue Phe118 in the SRD5A2 binding cavity could dramatically restrain the binding of testosterone [92]. Together with our findings, the residue Phe118 is the most crucial amino acid that is prone to interact with steroid substrates and ligands, including α-tocopherol.

By contrast, finasteride had strong hydrogen bond interaction with residues Arg94 and Glu57. The residue Phe118 interacted with finasteride via hydrophobic interaction (−4.5 kcal/mol) but less compared to its interaction with α-tocopherol. In accordance with our results, a previous study suggested that residues Glu57 and Arg114 interact with an intermediate adduct of finasteride and NADPH in the binding pocket [34]. In addition, residues Tyr235, Asp241, and Lys244 interact with finasteride inside the SRD5A2 binding cavity [91], but these residues were not significantly observed in this study.

Although β- and γ-tocopherol form a number of notable interactions with residues in the SRD5A2 binding pocket, the complexes were much less stable than α-tocopherol and finasteride. This may be due to fact that the chromanol ring of α-tocopherol contains three methyl groups at the C5-, C7-, and C8-positions, while β- and γ-tocopherol have two methyl groups. Consequently, the residues in the pocket are likely to form a hydrophobic interaction with these methyl groups of α-tocopherol, resulting in the stabilisation of ligands [93]. The rigid moieties at the phytyl tail of β- and γ-tocopherol might contribute to the steric hindrance effect and affect the binding pocket insertion [94].

We performed MD simulations to screen for promising SRD5A2 inhibitors and gain insight into the stability and overall dynamics of each ligand in the SRD5A2 pocket before the intermediate adduct is formed. However, the present study only provides a proof-of-concept by focusing on the molecular interactions of the ligand in the binding site without a membrane environment. Because SRD5A2 is a membrane-embedded steroid reductase, the influence of the membrane environment is an important factor for the protein conformational dynamics [95]. A previous advanced MD study has demonstrated that steroid substrates presumably access the SRD5A2 binding pocket from the lipid bilayer through the opening between TM1 and TM4. Furthermore, high conformation dynamics of the cytosolic region were observed during the NADP+/NADPH exchange [34]. Membrane–protein interactions may affect ligand entry, ligand binding modes, and SRD5A2 structural conformations. Thus, future MD studies of SRD5A2 should include the lipid membrane to evaluate its influence on the system. The experimental study of promising ligands towards SRD5A2 needs further investigation to confirm the inhibitory activity.

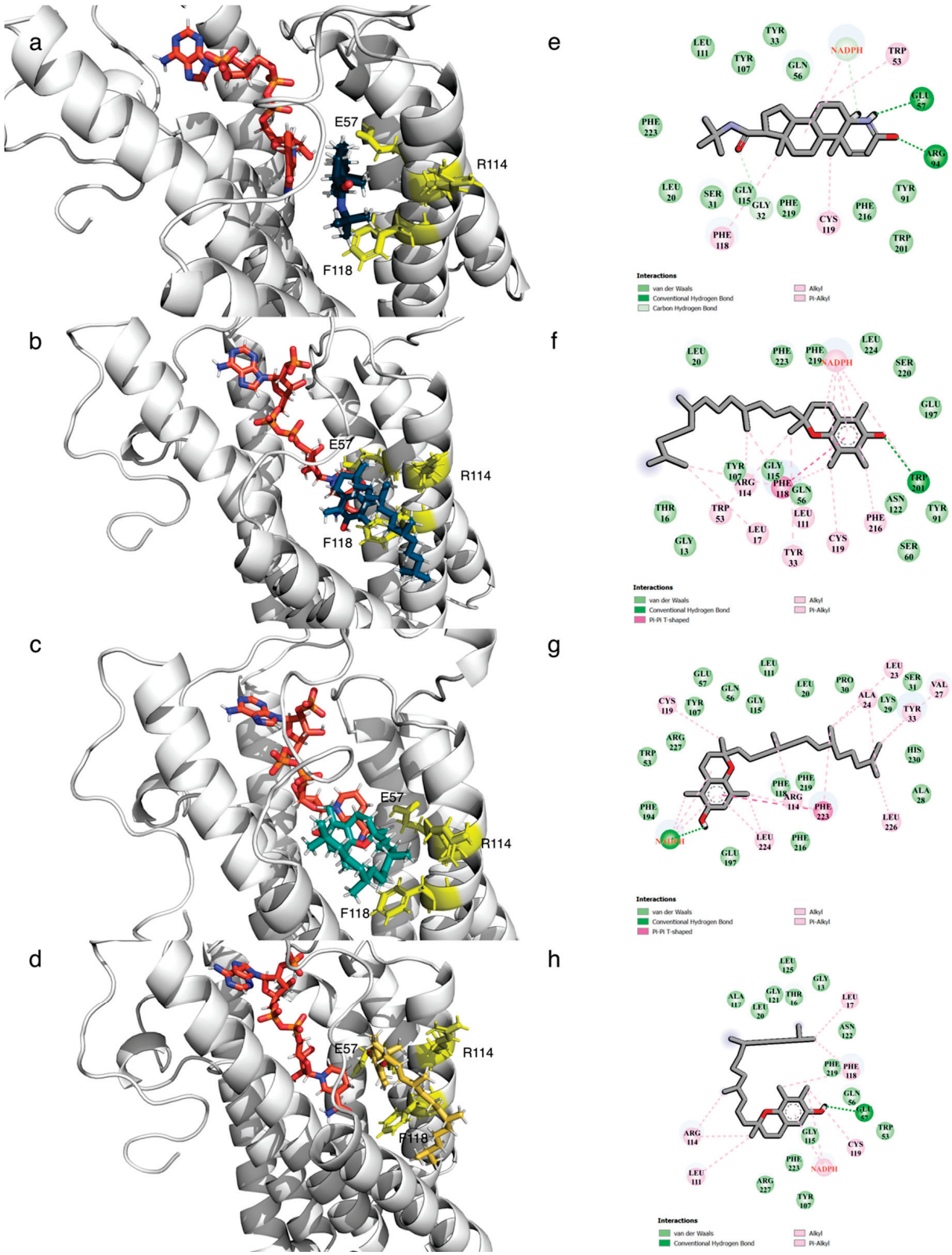

Figure 7. Molecular interaction patterns of 3D and 2D interactions of the ligands (finasteride and α-, β-, and γ-tocopherol) in the pocket of 5α-reductase type 2 after molecular dynamics simulation; the interacting residues are highlighted with different colours: (**a**,**e**) binding pose of finasteride, (**b**,**f**) binding pose of α-tochopherol, (**c**,**g**) binding pose of β-tocopherol, and (**d**,**h**) binding pose of γ-tocopherol.

4. Conclusions

Ethanolic TRB rice bran extract, a dark-brown semisolid extract, contained a decent amount of α-tocopherol and had the largest sum of overall bioactive compounds (γ-oryzanol; α-, β-, and γ-tocopherol; TPC and TFC). TRB also showed the highest antiandrogenic suppression of *SRD5A1*, *SRD5A2*, and *SRD5A3* mRNA. There was no statistically significant difference in the suppression of the mRNA expression of the three types of *SRD5A* between TRB and finasteride (FDA-approved for anti-hair loss treatment). The linear relationship and PCA indicated that the content of α-tocopherol shows a strong contribution to the suppression of all *SRD5A* genes. Strong evidence from previous studies indicates that SRD5A2 activity is implicated mostly in the reduction of T to DHT and expressed mainly in DPC obtained from hair follicles, highlighting the vital role of SRD5A2 in AGA development. SRD5A2 was subsequently docked with selected bioactive compounds based on the prior results of the studies indicating the involvement in androgen pathways. MD simulation demonstrated that α-tocopherol forms a stable interaction with SRD5A2 similarly to finasteride; it forms hydrophobic and hydrogen bond interactions with crucial amino acid residues in the SRD5A2 binding pocket, supporting the potential inhibition of this bioactive compound in TRB. Our findings bring new insights that TRB suppresses *SRD5A* gene expression as well as SRD5A2 activity. However, additional studies of enzymatic inhibition need to be evaluated. This will help to progress the uses of this rice by-product for future pharmaceutical and cosmeceutical applications as anti-hair loss products.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10040319/s1: Figure S1. Effects of selected bioactive compounds and rice bran extracts on steroid 5α-reductase isoenzyme (*SRD5A*) expression in DU-145 cells treated with 0.10 mg/mL of ethanolic rice bran extracts (TRB, YRB, RRB, and MRB), 0.01 mg/mL of selected bioactive compounds, and 0.10 mg/mL of standard controls (finasteride and dutasteride). (a) Suppression of *SRD5A1*. (b) Suppression of *SRD5A2*. (c) Suppression of *SRD5A3*. A statistical significance in comparison to controls is indicated as * $p < 0.05$; Table S1. The percentage of *SRD5A* suppression of four rice bran extracts and their bioactive constituents.

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Review

Beneficial Role of *Carica papaya* Extracts and Phytochemicals on Oxidative Stress and Related Diseases: A Mini Review

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Simple Summary: This review highlights the medicinal benefits of a natural remedy, the *Carica papaya* extracts and its phytochemicals. In this review, the potential of *Carica papaya* against various conditions, including cancer, inflammation, aging, healing of the skin, and lifelong diseases has been summarized and discussed. In short, more research and development should focus on this natural remedy that can potentially act as a prophylaxis against chronic diseases.

Abstract: Oxidative stress is a result of disruption in the balance between antioxidants and pro-oxidants in which subsequently impacting on redox signaling, causing cell and tissue damages. It leads to a range of medical conditions including inflammation, skin aging, impaired wound healing, chronic diseases and cancers but these conditions can be managed properly with the aid of antioxidants. This review features various studies to provide an overview on how *Carica papaya* help counteract oxidative stress via various mechanisms of action closely related to its antioxidant properties and eventually improving the management of various oxidative stress-related health conditions. *Carica papaya* is a topical plant species discovered to contain high amounts of natural antioxidants that can usually be found in their leaves, fruits and seeds. It contains various chemical compounds demonstrate significant antioxidant properties including caffeic acid, myricetin, rutin, quercetin, α-tocopherol, papain, benzyl isothiocyanate (BiTC), and kaempferol. Therefore, it can counteract pro-oxidants via a number of signaling pathways that either promote the expression of antioxidant enzymes or reduce ROS production. These signaling pathways activate the antioxidant defense mechanisms that protect the body against both intrinsic and extrinsic oxidative stress. To conclude, *Carica papaya* can be incorporated into medications or supplements to help manage the health conditions driven by oxidative stress and further studies are needed to investigate the potential of its chemical components to manage various chronic diseases.

Keywords: oxidative stress; antioxidant; *Carica papaya*; inflammation; diabetes; cancer; aging; wound healing; periodontal disease; Alzheimer's disease

1. Introduction

Oxidative stress is a natural phenomenon, resulting from the disruption of the redox equilibrium due to the amount of pro-oxidants outweighing antioxidants, which can eventually result in cell or tissue damage. As the name suggests, either oxidative stress can be induced by the presence of a high amount of pro-oxidants or the incompetence of antioxidant defense mechanism in the human body. In normal circumstances, human body is capable of scavenging free radicals, inhibiting the generation of oxidative stress with the help of several antioxidant enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT). Among various pro-oxidants, those free radicals that contain oxygen are known as reactive oxygen species (ROS), and ROS are considered as the secondary products of aerobic metabolism. Examples of ROS include singlet oxygen, hydroxyl radicals, superoxide radicals, and hydrogen peroxide. Sources of free radicals include diet, environment, and sunlight exposure and their accumulation can lead to oxidative stress and tissue injury, consequently leading to aging of the skin and medical conditions, including Alzheimer's disease (AD), rheumatoid arthritis, asthma, atherosclerosis, and cancers [1,2]. Various researches have been conducted to investigate the pathophysiology of diseases related to oxidative stress and the benefits of antioxidants in treating those diseases. Antioxidants can be found abundantly in plants. An example of antioxidant-rich plant is the *Carica papaya* L., which is a flowering and dicotyledonous plant, classified as violales order, *Caricaceae* family, *Carica* L. genus, and papaya species [3]. The *Carica papaya* L. has a single hollow light greenish to brownish stem with scarring, bearing big leaves and big oval fruits. Besides, this plant is cultivated in countries, such as Malaysia, Brazil, South America, Australia, and Indonesia, which are located near to the equator. The *Carica papaya* L. plant is known as many different names, such as kepaya, paw paw, or tapaya, based on its geographical distribution. In fact, this plant is acclaimed for an array of medicinal values from each part of the plant including fruit, roots, leaves, and seeds of the plant. Therefore it has been used as a traditional treatment regimen for various diseases [4]. Some of the medicinal properties of the plant can be explained by its antioxidative property, which confer protection on the cells from being harmed by oxidative stress [5]. Papain is the most widely exploited proteolytic enzyme from the *Carica papaya* L. and it has been used to help with meat tenderization and digestion. It is worth to note that papain exhibited great potential as a medication [4], as it is suggested to exhibit drug-like properties for atherosclerosis and associated conditions, which involve monocyte-platelet aggregate (MPA)-regulated inflammation [6]. Relevant and significant studies have been conducted to evaluate the benefits of the *Carica papaya* extracts and chemical constituents. This review aims to gather and summarize the research findings linking the *Carica papaya* to its antioxidant properties and the utilization of this natural resource as a pharmaceutical, cosmeceutical, and nutraceutical products.

2. Methods

All literature was retrieved from databases (PubMed, Semantic Scholar, Web of Science, WorldWideScience, and Embase) using search terms, including "*Carica papaya*", "inflammation", "cancer", "Alzheimer's Disease", "diabetes", "aging", "wound healing" and "oxidative stress". Literatures published from 2000 to 2020, investigating the benefits of the *Carica papaya* plant towards various conditions, were included. Literatures that were not related to oxidative stress mechanisms were excluded. Literatures selected were categorized based on related conditions including inflammation, cancer, skin aging, wound healing, diabetes, periodontal diseases, and Alzheimer's disease (AD). The mechanisms of action of the *Carica papaya* towards each condition were also presented in tables in the respective sections.

3. *Carica papaya* Counteracts Oxidative Stress in Inflammation, Skin Aging, and Healing, Chronic Diseases, and Cancers

Oxidative stress occurs due to excessive ROS production, which will cause oxidative damage to tissues. Consequence effects of oxidative stress has known to cause inflammation, leading to the development of various health conditions, including AD, rheumatoid disease, cardiovascular diseases (CVDs), cancers, cataracts, as well as cosmetic issues, such as the formation of wrinkles and loss of elasticity of the skin [7,8]. Figure 1 provides an overview of the role of oxidative stress in these conditions.

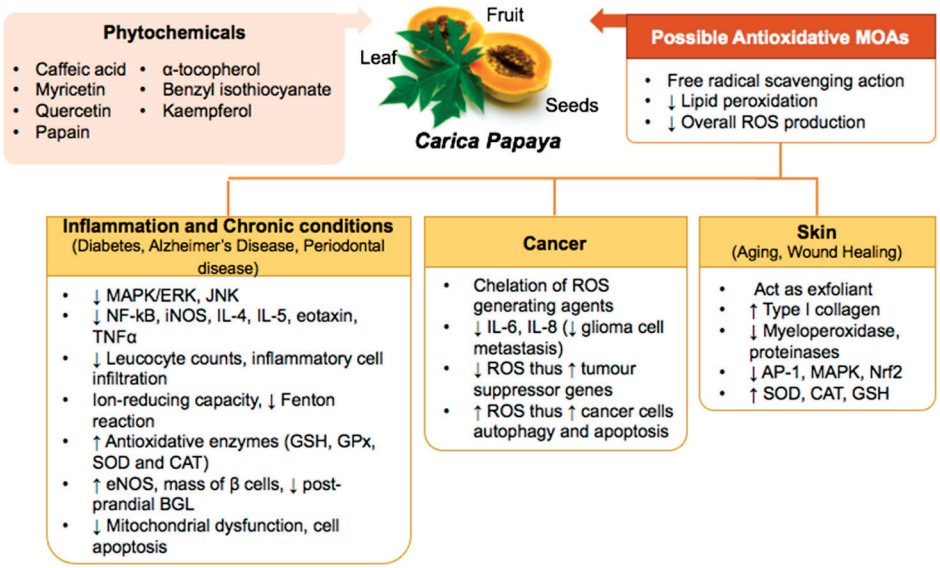

Figure 1. Role of oxidative stress in different medical conditions.

3.1. Inflammation

Inflammation is a complicated pathway of the body's own protective mechanism against pathogens, which is associated with symptoms such as pain, swelling, and redness due to the release of a mediator "prostaglandin" [9]. This defensive action can be divided into innate and adaptive responses [10]. In short, the pathogenesis of inflammation starts with tissue injury, which causes infiltration and activation of macrophages and relevant antigen-presenting cells (APCs). This causes the release of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukins (ILs). Cytokines stimulate the release of chemokines, which further recruit and activate lymphocytes and leukocytes. ROS are produced to eliminate invaders whereby activates Nuclear factor kappa-B (NF-κB). NF-κB is a transcription factor and plays a role in inducing inducible nitric oxide synthase (iNOS) activity and, thus, nitric oxide (NO) production. Excessive ROS upregulated prostaglandin E2 (PGE2) synthesis and, hence, cyclooxygenase-2 (COX-2) expression, which eventually leads to oxidative stress that causes tissue damage and worsens inflammation [11–13].

Another study further suggested that oxidative stress and inflammation are interrelated as oxidative stress resulting from high ROS can precipitate the formation of inflammation by increasing the gene expression coding for inflammatory proteins, including NF-κB, peroxisome proliferator activator receptor gamma (PPAR-γ), and activator protein 1 (AP-1). Consequently, inflammatory chemokines and cytokines are produced to induce inflammation. On the other hand, inflammation can increase ROS production via several signaling cascades. Polymorphonuclear neutrophils (PMN) is an immune cell that is largely involved in inflammatory processes. During inflammation, they congregate the gp91-phox, which is a catalytic subunit of NADPH oxidase 2 (NOX) and generate more ROS, including hydroxyl radical, superoxide anion, and hypochlorous acid, thereby enhance inflammation through mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and c-Jun-N-terminal kinase (JNK) pathways [14]. Activation of these signaling cascades lead to production

of more inflammatory chemokines and cytokines. Therefore, this forms a vicious cycle leading to chronic inflammation and eventually a range of medical conditions, including cardiovascular diseases, neurodegenerative diseases, and cancers [12,14].

Table 1 shows anti-inflammatory activities of *Carica papaya*. Different parts of *Carica papaya* possess anti-inflammatory effects. Aqueous extract of *Carica papaya* seeds significantly reduced NO radical by 69.4% in a cell free assay in vitro. Meanwhile, the aqueous extract at a concentration of 150 μg/mL inhibited the release of lysosomal enzymes and stabilized human red blood cell membrane by 22.7%. On the contrary, the extract exhibited least potent hydroxyl radical scavenging action (69.1%) at a concentration 95 mg/mL and reducing power at a concentration of 20 mg/mL [15]. Meanwhile, Aruoma and colleagues demonstrated that fermented papaya preparation (FPP) inhibited H_2O_2-induced phosphorylation of Akt and p38, as well as downregulating MAPK pathway [16]. An in vivo study showed that *Carica papaya* leaf extract at a dose of 1.32 μg/mL demonstrated immune modulation properties [17].

Table 1. Anti-inflammation activities of *Carica papaya.*

Part of the Plant	Extract	Type of Experiment	Results	Reference
Seed	Aqueous extract	In vitro cell free model In vitro HRBC assay	Aqueous extract of papaya seeds at 20 μg/mL decreased NO radical by 69.4%, comparable to ascorbic acid.	[15]
			Aqueous extract of papaya seeds at 150 μg/mL inhibited the release of lysosomal enzyme by 22.7%.	
Leaf	Leaf extract	In vitro animal model	Leaf extract at 1.32 μg/mL enhanced adaptive immune response by upregulated TLR-7 and TLR-9 expressions.	[17]
Fruit (ripe, unripe fruit, peel, seed, pulp)	Aqueous extract	In vitro ROS assay	Unripe peel (69.7%) and seed (79.1%) extract at 2mg/mL showed ROS scavenging activity of 69.7 and 79.1% at 2 mg dry weight/mL.	[18]
		In vitro antioxidant enzyme assay	Aqueous extract of papaya unripe peel at 2 mg dry weight/mL increased SOD activity by 21.9%.	
		In vitro protein carbonyl assay	Aqueous extract reduced oxidative damage by lowered protein carbonyl production for ripe seed (60.4%) and ripe peel (57.6%) extract at 0.1 and 2 mg/mL.	
		In vitro inflammatory cytokines assay	The extracts augmented IL-10 levels at a low concentration of 0.1 mg/mL. Seed extracts exerted highest increment in IL-10 secretory level (+140.1%), followed by peel and pulp extracts. Seed extracts at 0.1 mg dry weight/mL exerted increment in IL-10 secretory level (+140.1%).	
			Aqueous extract of papaya seeds at 2 mg dry weight/mL down regulated IL-6 by 37.8%.	
			Unripe extracts at 2 mg/mL showed inhibitory activity against TNF-α with 71.2% for pulp extract, 62.7% for peel and 65.3% for seed extract.	

Table 1. *Cont.*

Part of the Plant	Extract	Type of Experiment	Results	Reference
Fruit (Flesh)	Juice	In vitro animal model	Papaya juice downregulated the elevated serum IL-6 (217.6 vs. 28.3 pg/dL) and MDA (3.2 vs.1.4 pg/dL) in high fat diets treated rats. Papaya juice affected serum SOD of the high fat treated rat by increased serum SOD (30.41 U/L).	[19]
Leaf	Ethanol extract	In vitro animal model	Ethanol extract of papaya leaves at 200 mg/kg reduced paw edema (2.6 mm) and inhibited granuloma formation (0.2 g).	[20]

Abbreviation: NO, nitric oxide; HRBC, human red blood cell; TLR, toll-like receptors, MDA, serum malondialdehyde.

Somanah and co-workers revealed that papaya extracts at a dose of 2 mg/mL showed protective effects through attenuated ROS production and pro-inflammatory cytokines secretion of interleukin-6 (IL-6) and TNF-α as well as upregulating antioxidant enzymes activities [18]. Another in vivo study showed that papaya juice demonstrated anti-obesity properties by reducing obesity markers, inflammation and oxidative stress in high-fat diet rats by upregulating SOD levels, attenuated serum malondialdehyde (MDA), PPAR-γ, lipid peroxidation, and ROS production at a treatment dose of 1 mL per 100 g of body weight [19].

The anti-inflammatory effect of *Carica papaya* was further investigated on various in vivo experimental studies. For instance, ethanolic extract of *Carica papaya* leaves was found to reduce paw edema induced by carrageenan, granuloma formation, as well as inflammation in formaldehyde-induced arthritis rats at doses of 25–200 mg/kg [20]. Methanolic extract of *Carica papaya* seeds at dosage range of 50 to 200 mg/kg exhibited anti-inflammation activities in egg albumin induced inflammation on Wistar albino rats [21]. Similarly, the aqueous extract of *Carica papaya* seeds at a dose of 400 mg/kg showed anti-inflammatory in carrageenan and formalin induced pedal edema rats [22].

High phenolic and flavonoid content in papaya seed extracts were proposed to act as free radical scavengers and metal ion chelators [15]. Phytochemicals including tocopherols and quercetin are showed to enhance AMP-activated protein kinase (AMPK) activation, as well as the inhibition of COX-2 expression [17]. In addition, a range of phytochemicals with great strength of anti-inflammatory effect, such as benzyl isothiocyanate (BiTC), β-carotene, lycopene, and vitamin C could be found in various parts of papaya fruits, in either pulp or seeds. These phytochemicals were proven to inhibit pro-inflammatory cytokines including TNF-α, IL-6 and monocyte chemoattractant protein-1 (MCP-1) [18,19]. In addition, polyphenols within *Carica papaya* could act as free radical scavenger and at the same time exerting its effects in upregulating the antioxidant enzymes activities [18].

3.2. Diabetes

Diabetes is a chronic disease, predominantly due to the insulin resistance or insulin insufficiency phenomenon, which leads to elevation of blood glucose level, a condition known as hyperglycemia [23]. Uncontrolled diabetes can lead to various macro and microvascular complications in which ultimately affect the quality of life of diabetes patients. It has been shown that oxidative stress plays an important role in diabetes and its progression [24]. There are tremendous amount of evidences revealed that uncontrolled hyperglycemia might induce oxidative stress by promoting ROS production and weakening antioxidant defenses via several mechanisms, including inducing lipid peroxidation of low-density lipoprotein (LDL), glycation of proteins, and glucose oxidation. Non-enzymatic interaction of glucose with proteins generates advanced glycation end products (AGEs), and increases nitric oxide (NO). Excessive free radicals can cause dysfunction of β-cells of the islets of Langerhans of pancreas and lead to complications. Thus, these findings support the role of antioxidants in diabetic control [25,26]. Table 2 shows anti-diabetic activities

of *Carica papaya*. Agada and co-workers demonstrated that the ethyl acetate extract of *Carica papaya* seeds significantly reduced postprandial glucose levels in streptozotocin-induced diabetic rats. Along with in vivo study, ethyl acetate showed α-glucosidase and α-amylase enzyme inhibitory effect and antioxidant activities in vitro, whereas hexane extract exhibited slightly more potent enzyme inhibitory activities [27].

Table 2. Anti-diabetes activities of *Carica papaya.*

Part of the Plant	Extract	Type of Experiment	Results	Reference
Seed	Hexane extract & ethyl acetate extract	In vitro DPPH radical scavenging assay	Hexane extract possessed DPPH radical scavenging activity with IC_{50} = 41.5 mg/mL.	[27]
		In vitro TBA method	Hexane extract demonstrated TBA scavenging activity with IC_{50} = 38.2 mg/mL.	
		In vitro α-glucosidase inhibition	Hexane extract displayed α-glucosidase enzyme inhibitory activity with IC_{50} = 75.78 mg/mL.	
			Ethyl acetate extract exhibited α-glucosidase enzyme inhibitory activity with IC_{50} = 77.41 mg/mL.	
		In vitro α-amylase inhibition	Hexane extract demonstrated α-amylase inhibitory activity with IC_{50} = 76.96 mg/mL.	
			Ethyl acetate extract displayed α-amylase inhibitory activity with IC_{50} = 79.18 mg/mL.	
		In vitro FRAP assay	Ethyl acetate extract displayed FRAP inhibitory activity with IC_{50} = 38.75 mg/mL.	
		In vitro animal model	Ethyl acetate extract at 500 mg/kg/body weight significantly decreased the blood glucose level of the diabetic rats to approximately 120 mmol/L over 120 min comparable with standard drug, acarbose.	
-	FPP	In vitro analysis	FPP at concentration 50 µg/mL increased inner and outer platelet membrane fluidity, displayed by a decrease of ~0.015 r in DPH anisotropy and ~0.02 r in TMA-DPH anisotropy. FPP increased Naþ/Kþ-ATPase activity by ~0.5 µmol Pi/mg prot/h.-FPP improved platelet function in vitro and this might help preventing diabetic complications. FPP also slightly increased TAC by ~5 nmol/µL and SOD activity by ~0.5 units/µL. FPP at 50 µg/mL lowered lipid peroxidation.	[28]
-	FPP®	Human trial	FPP significantly improved liver sensitivity to insulin, which was indicated by decreased circulating AST and ALT. FPP scavenged NO and hydroxyl radicals and displayed an increased in total antioxidant status.	[29]

Table 2. *Cont.*

Part of the Plant	Extract	Type of Experiment	Results	Reference
-	FPP	In vitro DPPH radical scavenging assay	FPP displayed DPPH scavenging with AA_{50} = 55.69 mg/mL.	[30]
		In vitro $ABTS^+$ scavenging assay	FPP demonstrated $ABTS^+$ scavenging action with AA_{50} = 14.56 mg/mL.	
		In vitro AAPH-induced lipid oxidation inhibition	FPP inhibited AAPH-induced lipid oxidation with AA_{50} = 68.06 mg/mL.	
		In vitro O_2^- scavenging assay	FPP showed O_2^- scavenging action with AA_{50} = 88.70 mg/mL.	
		In vitro •OH scavenging assay	FPP showed hydroxyl radical scavenging activity with AA_{50} = 4.13 mg/mL.	
		Human trial	FPP at a dose of 6g/day showed an increase of 4.9% and 5.7% in TAS for male and female respectively after 14-week consumption at 6g/day. FPP decreased protein carbonyl level by 1.9% in males and 9.7% in females after a 14-week FPP ingestion. FPP delayed red blood cell hemolysis.	
Seed, flesh and peel of unripe fruit	Aqueous extract	In vitro α-amylase inhibition In vitro α-glucosidase inhibition In vitro lipid peroxidation assay In vitro NO scavenging assay	Aqueous extract inhibited α-glucosidase and α-amylase activities with IC_{50} of 1.76 mg/mL and IC_{50}: 0.87 mg/mL. At a concentration of 7.5 mg/mL, the extract also displayed the highest NO radical scavenging activity (52.5%).	[31]
Leaf	Chloroform extract	In vitro animal model	The chloroform extract at a dose of 31 mg/kg/day significantly increased islet area by 16,842.2 μm^2 by stimulating regeneration of β-cells of islet of Langerhans.	[32]
			The extract successfully decreased fasting glucose levels by 222.3 mg/dL in diabetic group in vivo.	
Leaf	Aqueous extract	In vitro animal model	Aqueous extract at a dose of 3 g/100 mL decreased blood glucose levels in diabetic rats by 184 mg/dL. Aqueous extract at a dose of 1.5 g/100 mL preserved Islet cell size in diabetic rats. Aqueous extract increased NO levels by 17.39 μM and hence reduced ROS production.	[33]

Abbreviation: DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; TBA, thiobarbituric acid; FPP, fermented papaya preparation; TAC, total antioxidant capacity; SOD, superoxide dismutase; AST, aspartate transaminase; ALT, alanine aminotransferase; NO, nitric oxide. Footnote: IC_{50} = concentration needed for 50% inhibition; AA_{50} = concentration needed to achieve 50% antioxidant activity.

Carica papaya FPP extract showed protective effect against diabetic complications such as atherosclerotic plaque formation, upregulated SOD level and ameliorated lipid peroxidation at a concentration of 50 μg/mL. In addition, increasing platelet membrane fluidity of diabetic patients and preventing chronic hyperglycemia-induced platelet malfunction [28]. Likewise, Somanah and colleagues conducted a study on the impact of short-term supplementation of fermented papaya preparation (FPP) on the biomarkers of diabetes mellitus. The randomized controlled trial showed that daily consumption of 6 g of FPP for a period

of 14 weeks enhanced the antioxidant status of the subjects and improved general health status of several organs that were potentially at risk of damage from diabetes. In addition, the FPP extract reduced the aspartate transaminase (AST) and alanine aminotransferase (ALT) levels to enhance insulin sensitivity of the liver and stabilize blood glucose level in diabetic patients [29]. Furthermore, a continuous human trial conducted by Somanah and co-workers showed that the supplementation of FPP successfully reduced erythrocyte hemolysis rate in pre-diabetics. FPP possessed hydroxyl-quenching properties that could possibly prevent DNA damage and boost the total phenolic content that exhibited antioxidant activities [30].

Unripe papaya has been used as a folk medicine, e.g., to relieve menstrual pain, improve ingestion, wound healing, and heart disease. An in vitro study showed that unripe *Carica papaya* fruit inhibited α-amylase and α-glucosidase enzymes. In addition, the fruit extract protected β-cell against oxidative stress in streptozotocin induced diabetes rats. The phytochemical analysis of *Carica papaya* fruits revealed the presence of phytochemicals, including kaempferol, quercetin, and caffeic acid [31].

An in vivo study suggested that chloroform extract of *Carica papaya* leaves protected β-cells of islet of Langerhans from oxidative stress-induced damage and promoted pancreatic β-cells regeneration at a dose of 31 mg/kg, leading to an increase in insulin production [32]. In addition, Juarez-Rojop and colleagues reported that *Carica papaya* leaf extract stimulated the healthy β-cells to release more insulin in vivo. At concentrations of 0.75 and 1.5 g/100 mL, *Carica papaya* leaf aqueous extract also demonstrated antioxidant properties via increasing NO production, consequently lowering ROS production, and diminishing diabetes-induced oxidative stress. As a result, this mechanism delayed or prevented the progression to diabetic complications, such as neuropathy and nephropathy [33].

3.3. Alzheimer's Disease (AD)

Oxidative stress is correlated with the induction and progression of Alzheimer's disease (AD). AD is manifested by generation of neurofibrillary tangles and an aggregation of β-amyloid peptides in the brain [34]. As the amount of β-amyloid accumulates, it generates ROS that causes lipid and protein peroxidation in the brain, and resultant in neurotoxicity. In an AD brain, there is an impairment of the defense mechanism against oxidative stress due to a reduction in the concentration of glutathione. Furthermore, generation of ROS in the brain inhibits the activity of α-secretase whilst promoting the activity of γ- and β-secretase via generation of neurotoxic β-amyloid 40 and 42 [35]. These two mechanisms form a vicious cycle in the AD pathology. Another mechanism notably suggested that β-amyloid impairs mitochondrial function of neuronal cells in AD patients; therefore, promoting neuronal cell death by inducing oxidative injury in isolated mitochondria. β-amyloid impairs the antioxidative stress mechanism by lowering the expression of uncoupling proteins (UCPs) that act to promote mitochondrial uncoupling and reduce ROS generation [35]. A limited number of studies proposed that oxidative stress susceptibility is increased by overexpression of tau protein in neuronal cells. In addition, presence of transition metals including iron, zinc, and copper can react with the β-amyloid to produce hydrogen peroxide (H_2O_2) in the brain [34–36].

Fermented papaya preparation (FPP) is a popular health-promoting product which owns protective properties against free radicals to improve general health. Meanwhile, fermented papaya preparation (FPP) exerted neuroprotective properties against copper induced neurotoxicity in Swedish mutant human APP (APPsw) cells at a dose of 2.4 mg/mL by reducing 64% of ROS generation. In addition, FPP significantly reduced scavenging of superoxide anion and hydroxyl radicals and upregulation of SOD-1 enzyme. FPP exerted anti-apoptotic effect and attenuated pro-apoptotic Bax gene expression, upregulated BCL level, and maintained calcium homeostasis, leading to improvement of neuronal cell survival and AD condition. Administration of 2.4 mg/mL FPP inhibited up-regulation of expression of iNOS, nNOS, and NO by about 43%, 71%, and 40%. Moreover, treatment with 2.4 mg/mL FPP lowered the secretion of Aβ peptide by 30.6% [37].

FPP significantly reduced the 8-hydroxy2′-deoxyguanosine (8-OHdG) level in AD patients treated with FPP at a dose of 4.5 g/day for 6 months. During the study period, no neurotrophic drugs were administered to the study participants; therefore, proving the value of the *Carica papaya* plant in improving AD. The proposed mechanisms of action by FPP include decreased peroxidation of lipids, aluminum and iron induced neuronal toxicity and free radicals' production [38]. Overall, FPP showed promising anti-Alzheimer's disease in cell-based model and human trial. Studies including discovery of novel phytochemicals, safety profile, and efficacy warrants future investigation.

3.4. Periodontal Disease

Periodontal disease is an infection on the tissue that supports the tooth, which is closely related to oxidative stress. Several examples of periodontal diseases include gingival inflammation, chronic periodontitis, aggressive periodontitis, necrotizing periodontal disease and periodontal associated lesion. These conditions can happen to anyone ranging from a juvenile to an adult [39]. When the integrity of tissues supporting the tooth is compromised, the immune response of the host is triggered secondary to pathogen invasion and eventually led to inflammation. Periodontal inflammation can be augmented by excessive ROS and leukocytes and damaging the periodontal tissues. Likewise, periodontal tissue injury also occurs when there is a disruption in the redox equilibrium, due to either over-generation of ROS or diminished antioxidant enzymes, including GPx, CAT, and SOD, which defend against oxidative stress. ROS plays a role in activating signaling pathways, such as NLRP3 inflammasomes, NF-κB, and JNK, which eventually lead to inflammation and cell death [40]. Human trial has shown that *Carica papaya* leaf extract significantly alleviated gingival bleeding and inflammation [41].

Kharaeva and colleagues reported that the standardized fermented papaya gel (SFPG) application at 7 g/day for 10 consecutive days significantly reduced gingival inflammation and bleeding in participants by decreasing nitrate (NO_3^-) and nitrite level (NO_2^-), and regulating the level of inflammatory cytokines. Reduced NO_3^- and NO_2^- attenuated production of peroxynitrite and oxidative stress generation. Furthermore, the antioxidant effect was reported to last as long as 35 days after stopping SFPG application. Interestingly, SFPG was able to augment bacterial killing by impeding activation of bacterial catalase and eventually prevent infection at the periodontal sites [42].

Studies have also shown the protective effects of *Carica papaya* leaf extract dentifrice on interdental gingival bleeding. Participants who used dentifrice containing *Carica papaya* leaf extract demonstrated a significant decrease in the gingival bleeding and inflammation especially in advanced (>70%) gingival bleeding cases. This result could be attributed to the high phenolic content of *Carica papaya* leaf extract that possess antioxidant properties. A study revealed that *Carica papaya* leaf extract exerted anti-inflammatory action by decreasing TNF-α [41].

3.5. Skin Aging

Skin aging is characterised by extracellular matrix (ECM) degradation in which human skin naturally becomes drier, thinner, unevenly pigmented, and wrinkled, as a human being ages, due to the inevitable intrinsic aging factors. Extrinsic aging factors are avoidable, in which both factors may synergize and lead to premature skin aging. ROS is known to be the culprit of skin aging by contributing to oxidative stress and inflammation. Photoaging is a process that produces ROS, which eventually leads to augmented ECM turnover and degradation. Although not fully deleterious to the cells, excessive ROS can oxidise skin proteins and lipids leading to roughen the skin by altering the function of the skin barrier and further stimulate wrinkle formation [43].

In addition, ultraviolet (UV) and UV-generated ROS hasten aging via activation of mitogen-activated protein kinase (MAPK), p38, Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK), recruitment of c-Fos, and c-Jun, as well as increased expression of activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB). AP-1 is known to

lower transforming growth factor-beta (TGF-β), which is responsible for collagen production and induce expression of matrix metalloproteinase (MMP) 1, 3, and 9 in keratinocytes, and fibroblast leading to the disruption and loss of ECM components (collagen and elastic fibers) [43]. UV and ROS causes the skin to be in the state of "sunburn" (erythema). This further stimulates production of advanced glycation end products (AGEs). Activation of receptor for AGEs (RAGE) increases NF-κB activation, thereby upregulates pro-inflammatory gene transcriptions and RAGE leading to a vicious inflammatory state cycle characterised by elevated PGE2 synthesis [43,44].

Furthermore, ROS induces melanogenesis by increasing the number of tyrosinase-related protein 1 (TYRP-1) and tyrosinase, which are both known as melanogenic factors resulting in skin pigmentation [44]. In addition, UV radiation induced greater amounts of oxidised lipids, triglyceride hydroperoxides, and cholesterol hydroperoxides generation, leading to increased sebum secretion. This condition in turn promotes the formation of acne vulgaris by *Propionibacterium acnes* (*P. acnes*). *P. acnes* infects skin cells and will further induce the production of free oxygen radicals that eventually lead to the formation of inflammatory lesions [44].

In past decades, research on strategies against skin aging attracted a great attention of researchers. For instance, *Carica papaya* is a potential candidate to be exploited for its anti-skin aging specialty, owing to its antioxidant and anti-inflammatory activities. Table 3 shows anti-skin aging activities of *Carica papaya* extracts. An in vitro study by Jarisarapurin and colleagues focused on unripe *Carica papaya* fruit extract against skin aging related endothelial oxidative stress [45]. It was proposed that activated endothelial cells contributed to a low-grade inflammatory state and the generation of oxidative stress. As a result of this unfavorable microenvironment, MMP-1 expression in dermal fibroblasts was induced leading to a significant loss of type I collagen, and accelerated ECM degradation [46]. The study demonstrated the ability of unripe *Carica papaya* fruit extract to inhibit H_2O_2-induced endothelial cell death at concentrations ranging from 100 to 1000 μg/mL. It was found to exert its effect via modulating intracellular stress and antioxidant defenses in endothelial cells. The mechanisms were consisted of a dose-dependent ROS scavenging effect and NF-κB attenuation, upregulation of SOD and CAT activities, and prevention of H_2O_2-induced Nrf2 over activation. The study further explained that, although activation of antioxidant defenses was prompted by uncoupling of the Nrf2/Keap1 complex, followed by translocation of Nrf2 into the nucleus, the early (or over activation) of Nrf2 induced by oxidative stress can lead to depletion of endogenous antioxidants. The consequence of depletion of natural antioxidants produced by skin cells may promote skin aging. Therefore, the restraining properties of unripe *Carica papaya* on NF-kB elevation and Nrf2 dysregulation were proposed to be beneficial in maintaining redox homeostasis, thereby delaying skin aging [45].

A recent study by Seo and colleagues investigated the anti-aging mechanisms of *Carica papaya* leaf ethanol extract on UVB-irradiated human dermal fibroblast cells in vitro. At concentrations ranging from 10 to 250 μg/mL, the extract demonstrated radical scavenging and ROS suppressing action in a dose-dependent manner. At concentrations of 1 to 50 μg/mL, the extract was shown to enhance synthesis and attenuate degradation of type I procollagen in UVB-irradiated fibroblasts, increment in TGF-β1, and reduction in MMPs (MMP-1 and MMP-3) generation. Interestingly, Seo and colleagues further evaluated that the leaf extract possessed reversal action on UVB-induced AP activation at mRNA level via downregulating MAPK activation and protein phosphorylation of c-Fos and c-Jun. The effect of *Carica papaya* leaf extract on MAPK was proposed to act mainly on p38, showing 82% inhibition against p38 phosphorylation, followed by ERK and JNK. The extract demonstrated to acquire anti-inflammatory action by depleting production of cytokines, such as IL-6. Wrinkles formation induced by sun exposure as a result of erythema and diminished Type I collagen in skin. The ROS-conquering mechanisms and collagen synthesis promoting effects were described by Seo and colleagues, lending support on the potential use of *Carica papaya* leaf extract against skin aging [47].

Table 3. Anti-skin aging of *Carica papaya*.

Part of the Plant	Extract	Type of Experiment	Results	Reference
Unripe papaya juice		In vitro antioxidant enzyme assays	Papaya juice at 1 mg/mL enhanced SOD (49%) and CAT (40.5%) activities.	[45]
		Western blot analysis	Papaya juice at 1 mg/mL restrained NF-κB translocation to nuclei and downregulated Nrf2 levels.	
Leaf	Ethanol extract	In vitro DPPH assay	Ethanol extract at 250 μg/mL showed ROS scavenging effect at 60%.	[47]
		In vitro DCFH-DA assay	Ethanol extract at 50 μg/mL showed potent suppressing action towards UVB-induced ROS production (60%).	
		In vitro MMPs and inflammatory cytokines production	Ethanol extract at 50 μg/mL of *Carica papaya* leaves enhances synthesis and prevents degradation of type I collagen via upregulating TGF-β1 and down-regulating MMP-1 (34% at 50 μg/mL), MMP-3, and IL-6 generation. Ethanol extract at 50 μg/mL of *Carica papaya* leaves reduced mRNA level of MMP-1 (56.8%) and type I procollagen (288.8%).	
		Western blotting assay	Ethanol extract at 50 μg/mL showed AP-1 activation via down-regulating c-Fos (89%) and c-Jun (44%) phosphorylation. Ethanol extract at 50 μg/mL attenuated MAPK activation, and p38 phosphorylation (82%), followed by ERK, and JNK phosphorylation.	
	FPP	Double-blinded RCT	FPP at a dose of 4.5 g showed anti-skin aging by demonstrating overall higher skin moisturization, elasticity, and surface evenness. FPP at a 4.5 g inhibited MDA production and up modulation of AQP-3, enhanced SOD and NO levels in FPP-treated group. FPP at 4.5 g downregulated pro-aging factors (CyPA and CD147 genes) suggesting to reduce risk of skin carcinogenesis.	[48]

Abbreviation: DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; DCFH-DA, dichloro-dihydro-fluorescein diacetate; SOD, superoxide dismutase; MMP, matrix metalloproteinase; RCT, randomized controlled trial, MDA, serum malondialdehyde.

A human trial by Bertuccelli and colleagues revealed that sublingual FPP 4.5 g sachet twice daily lowered biomarkers of skin aging. While both treatments attenuated skin MDA level, FPP showed superior anti-aging effects than antioxidant cocktails [45]. In addition, FPP elevated levels of SOD, NO, aquaporin-3 (AQP-3), and down-modulation of pro-aging cyclophilin-A (CyPA) and CD147 genes. The study proposed that the regulating effects of FPP on AQP-3 and pro-aging factors were crucial for significant improvement in skin health [48].

The potentiality of *Carica papaya* being formulated as cosmetic products was demonstrated by Saini and colleagues, as the ideal oil-in-water *Carica papaya* fruit cream prepared was uniform, stable, and had a shiny and smooth texture. This study further proved ROS suppression as the main mechanism of *Carica papaya* fruit against anti-aging, in which the 5% cream was potent, owing reducing power against H_2O_2 free radicals [49].

Flavonoids and phenolic acids were found in *Carica papaya* leaf and fruit extracts [50,51]. Flavonoids in *Carica papaya* are mainly kaempferol, myricetin, quercetin, and their glycosides, phenolic acids, such as caffeic acid and ferulic acid, are the key ROS suppressors

and antioxidant that displayed radical scavenging and metal chelating potential [50,52]. Caffeic acid and rutin were detected and proposed to be the main anti-skin aging components. Both phytochemicals were reported to downregulate MMPs expression and photoprotective against collagen degradation. Caffeic acid mitigated skin erythema via inhibitory action towards NF-κB and AP-1 signaling [48]. The ability of caffeic acid in film formulation to permeate and retentate in epidermis (stratum corneum) and dermis layer enhance its efficacy [50,53]. Besides, the anti-skin aging role of rutin was supported by a human trial, which showed enhanced skin elasticity and less wrinkles in individuals treated with rutin-containing cream. The findings of elevated type I collagen via lowering MMP expression and potent ROS scavenging in human dermal fibroblast cells further supported the anti-skin aging of *Carica papaya* chemical constituents [48,54].

Albeit several mechanisms were compiled and proposed, however, studies regarding the *Carica papaya* anti-skin aging effect were scarce. More evidence regarding various parts, therapeutic range, and the relevant phytochemicals of *Carica papaya* on skin aging are needed to ensure their efficacy.

3.6. Wound Healing

Wound healing is rather complex and well-coordinated with involvement of several stages of cellular responses, including inflammation, proliferation, and remodeling. The duration of each phase usually ranges from 1 to 4 days, 5 to 10 days, and 11 days onwards. Characterisation for each phase includes presence of leukocytes, angiogenesis, protein synthesis and deposition, epithelialization, wound contraction, and scar formation [55]. These processes can be altered by the presence of oxidative stress [56]. The efficiency of the wound healing process decreases with advancing age [57]. Oxidative stress can alter the speed of wound recovery as it depends on the amount of ROS present at the wound site. Although minimal ROS prevents infection, excessive ROS is known cytotoxic to fibroblasts and reduce flexibility of skin lipids. In addition, it also causes impairment to lipids, DNA, proteins, and cellular membranes, and subsequently, severely damages the tissue and promotes inflammation [56].

In the case of chronic wounds or impaired wounds, ROS production is excessive in response to NADPH oxidase (NOX) activation in macrophages and neutrophils during the inflammatory phase of the wound healing process, contributing to high oxidative stress that leads to the wound remaining not healed. Thereby, extracts and phytochemicals with great strength of antioxidative properties are beneficial in wound healing [58,59]. Another key factor for wound healing is the extent of inflammation level at different stages of healing. Inflammation is essential to prevent infection, stimulate angiogenesis, and matrix deposition via secretion of cytokines and angiogenic factors at the early stage of wound healing. However, excessive or prolonged pathological inflammation causes delayed wound healing and fibrosis. Hence, inflammation at certain phases is deemed crucial for wound healing, but not throughout the entire healing process [55,60].

It is known that oxidative stress plays a vital role in wound healing. Table 4 shows wound healing activities of *Carica papaya* extracts. The protective action of aqueous extract *Carica papaya* seeds against oxidative stress-induced apoptosis in human skin fibroblast further supported its role in wound healing. An extensive mechanistic study conducted found potent antioxidant action of the papaya extract against H_2O_2-induced oxidative stress specifically on fibroblast cells was activated via radical scavenging, reduction of calcium ions influx into cytoplasm, reversal of oxidative stress-induced mitochondrial dysfunction, and maintaining oxidative balance inside the cells [61]. Mikhal and colleagues showed that FPP possesses antioxidative stress and anti-inflammation activities. FPP inhibited superoxide (IC_{50} = 5 mg/mL), hydroxyl radicals (IC_{50} = 1.1 mg/mL), and total ROS (IC_{50} =2 mg/mL) in blood, as well as reduction in myeloperoxidase (MPO) and radical generation at wound sites in vivo [62].

In addition, topical application of 5 mg/mL *Carica papaya* fruit extract enhanced wound healing by exerting effect on regulation antioxidant enzymes, inflammation, and

arginine metabolism. The addition of an antioxidant, selenium to the regimen, further shortened the time for wound healing significantly and, hence, confirmed the mechanisms. Antioxidative stress related mechanisms include inhibition of lipid peroxidation, lower MDA level and enhanced expression of SOD, CAT, and GPx. *Carica papaya* fruit extract reduces inflammation associated with oxidative damage through upregulation of antioxidant enzymes, arginine metabolism, and cyclooxygenase specific inhibition in an excision wound model. The extract demonstrated an attenuated inflammatory state, increased collagen synthesis and vascularization at wound site. Transforming growth factor-beta (TGF-β), a cytokine that generates fibroblast recruitment was high at the inflammatory phase and reduced at the repairing phase. While expression of vascular endothelial growth factor A (VEGFA), an angiogenic factor was increased throughout the wound healing process. The further study showed that addition of selenium to the papaya fruit extract synergistically upregulated TGF-β and VEGFA resulting in a significant acceleration in the wound healing process [63,64].

An oral FPP supplementation at a dose of 0.2 g/kg body weight for 8 weeks was found to enhance diabetic wound closure via improved macrophages respiratory-burst function and iNOS production. Diabetic wounds are hard-to-heal due to being prone to infections as a result of compromised NO at the wound site. Another reason was the antibacterial effect of macrophages via NOX was downregulated by hyperglycemia, consequently, respiratory burst dysfunction was seen in diabetic patients. FPP was shown to reverse these conditions. Similar to previous report by Nafiu and colleagues, FPP supplement showed an increase in VEGFA expression, deemed as a crucial regulator in current scenario [65].

Dickerson and colleagues further examined the diabetic wound healing effect of FPP on type II diabetes mellitus patients. The participants were given oral FPP (9 g/day for 6 weeks), and showed that NADPH and cellular ATP level increased in human monocytic THP-1 cells treated with FPP. Besides, FPP also exhibited higher oxygen usage and mitochondrial membrane potential on monocytic cells, which further revealed its capability to correct the respiratory burst function, enhancing the defense mechanisms against pathogens in diabetics. FPP upregulated the mRNA expression of Rac2, which was essential for NOX activation and eventually enhancing respiratory burst in macrophages [66,67].

Meanwhile, Indran and colleagues investigated the protective effect of *Carica papaya* leaf aqueous extract against alcohol-induced hemorrhagic lesions. Pretreatment with 500 mg/kg leaf extract significantly reduced gastric ulcer index via reducing lipid peroxidation, MDA levels, and improving GPx activity at gastric mucosa. The study showed the radical scavenging activity, which might be contributed by polyphenols within leaf extract. It was suggesting that the alkaline content of the extract and its ability to neutralize stomach acidity, thereby protecting stomach against gastric ulcer [68]. The concepts were further supported by in vivo studies evaluating different parts of *Carica papaya* on incised, burned, and diabetic wounds respectively. The recent findings show that *Carica papaya* fruit and seed extracts demonstrated dose-dependent increment in hydroxyproline, fibrillation, epithelial thickness, shortened wound contraction, and epithelialization time [69–72].

Cysteine endopeptidases including papain and chymopapain showed wound healing activity that can be attributed to their proteolytic wound debridement and antibacterial effects [63,64,70,72]. This was established by an in vivo study using papain-based wound cleanser. The cleanser was formulated with 5 g of papain and α-tocopherol. The results showed superior collagen deposition and least fluid exudates compared to betadine cleanser leading to eschar reduction and quicker epithelialization [73]. Safety of *Carica papaya* extracts and dressings is of less concern as it is traditionally used to treat wounds and certain skin conditions [74]. Several studies further assured its safety to be used [61,67,71]. In addition, papaya dressing was safe to be used and compatible in hydrogel formulation [70,75].

Table 4. Wound healing activities of *Carica papaya.*

Part of the Plant	Extract	Type of Experiment	Results	Reference
Seed	Aqueous extract	In vitro cytoprotective assay	Aqueous extract of papaya seeds at 1mg/mL showed cytoprotective against H_2O_2 induced cell toxicity.	[61]
		Cell apoptosis assay	Aqueous extract of papaya seeds at a concentration of 1 mg/mL inhibited H_2O_2 induced apoptosis by approximately 30%.	
		MMP and Cytochrome C assay	Seed extract at 1 mg/mL inhibited oxidative stress-induced cell apoptosis, reduced mitochondrial dysfunction and impeded release of cytochrome C.	
		Western blot analysis	1 mg/mL of seed extract decreased overexpression of HSP-70 in fibroblasts.	
-	Fermented papaya (Biorex)	In vitro HRBC model	Biorex inhibited superoxide (IC_{50} = 5 mg/mL), hydroxyl radicals (IC_{50} = 1.1 mg/mL), and total ROS (IC_{50} = 2 mg/mL) in human red blood cells.	[62]
		In vitro animal model	Biorex (1–5 mg/mL) decreased the elevated radical generation in rats with burn trauma. Biorex reduced local inflammation and catalase activity.	
Unripe pulp	Papaya extract +/− Selenium	In vitro animal model	Papaya extract alone (PE) or with selenium (PES) enhanced wound closure in rats. Both PE and PES augmented SOD, CAT, and GPx activities. PE with selenium ameliorated oxidative damage at the wound site. PE enhanced wound healing via attenuating excessive inflammation, reduced COX-2, and MPO enzyme activity. PE and PES increased NO content by increasing iNOS, stimulating collagen deposition and angiogenesis. PE suppressed arginase activity during wound healing as indicated by decreased wound urea content.	[63]
Unripe papaya pulp	Papaya aqueous extract. Or Papaya PBS extract + Selenium	In vitro animal model	Total protein content (95.14 ± 1.15 mg/g tissue) in wound tissue was significantly higher in rats treated with PES at a dose 5 mg/mL twice daily for papaya and 0.5 µg/20 mL for selenium. Rats treated with PES demonstrated elevation in wound hydroxyproline (*55.15 ± 1.06 µg/mg), hexuronic acid (*60.84 ± 6.08 mg/g), and hexosamine (*35.23 ± 4.95 mg/g) contents. Overall reduced in migration of polymorphonuclear monocytes and increased fibroblast recruitment at wound sites. PE enhanced collagen synthesis and vascularization. Time required for wound closure was shortened, indicated by earlier increment in VEGFA and TGF-β1 expression.	[64]

Table 4. *Cont.*

Part of the Plant	Extract	Type of Experiment	Results	Reference
-	FPP	In vitro animal model	FPP at a dose of 200 mg/kg s improved wound closure via increasing ROS (superoxides) production by macrophages at wound site and promoting NO production at ~60%. Increased NO and ROS to support redox signaling and angiogenesis. FPP increased CD68, VEGF transcription, macrophages recruitment to wound site and promoted optimal angiogenesis environment.	[65]
Leaf	Aqueous extract	In vitro animal model	Aqueous extract of papaya leaves at a dose of 500 mg/kg protected the stomach from absolute ethanol induced injury. Aqueous extract decreased MDA levels by 0.031 μmol/L and increased GPx by 0.246 U/mg protein.	[68]
Fruit	Aqueous extract	In vitro animal model	Aqueous extract of papaya fruit significantly shrank the wound area at 100 mg/kg by 77% by increasing epithelialization rate, weight of dry and wet granulation tissues and promoting enzymatic debridement of wound. Aqueous extract-treated wound showed rapid collagen turnover and accumulation that enhanced wound healing.	[69]
Tree	Dried latex incorporated into hydrogel	In vitro animal model	Topical application of the dried latex-containing hydrogel (1–2.5%) increased hydroxyproline content. Significant wound contraction after application of this hydrogel day 12 at concentration of 2.5% and on day 20 at both concentrations of 1.0% and 2.5%.	[70]
Seed	Ethanol extract	In vitro animal model	Ethanol extract of papaya seeds at a dose of 50 mg/kg significantly reduced wound area by 88.96%. Ethanol extract produces well-organized collagen deposition and significant fibroblast activity.	[71]

Abbreviation: MMP, matrix metalloproteinase; HRBC, human red blood cell; HSP-70, heat shock protein 70; NO, nitric oxide; ROS, reactive oxygen species; COX-2, cyclooxygenase-2; MPO, myeloperoxidase; VEGFA, vascular endothelial growth factor A; TGF, transforming growth factor; FPP, fermented papaya preparation; MDA, serum malondialdehyde.

3.7. Cancers

Cancer is a prevailing topic and there is no absolute cure to date for various types of cancers. ROS generation as a result of metabolic reactions in the mitochondria plays a role in both initiation and potentially elimination of cancers. With a low amount of ROS that is tolerable by the body cells, the progression of cancer could occur through either promoting genomic DNA alterations or DNA damage that alters the normal physiological signaling pathways. For instance, mitogen-activated protein kinase (MAPK) activation, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) phosphorylation, and cyclin D1 expression are correlated to cancer progression and survival [76]. In the normal healthy cells, a significantly high level of ROS can lead to cellular damage and eventually cell death [77]. However, cancer cells generally have a higher resistance to oxidative stress than normal cells to allow for uncontrolled proliferation and to compensate for the survival

of cancer cells during metastasis from their site of origin [76]. However, increasing ROS to a specific threshold level, specifically for cancer cells is proven to attenuate cancer cell growth and progression.

Several studies showed the correlation of microRNAs and oxidative stress in the progression of cancer. The recent advancement of genomic studies has showcased the presence of certain groups of microRNAs may promote cancer cell proliferation and progression. For example, there is an overexpression of miR-210 detected in hepatocellular and breast carcinoma under hypoxia. miR-210 acts to regulate the ROS production and mitochondrial function by promoting cancer cell adaptation, survival, and proliferation [77]. It also suggested that ROS is able to induce carcinogenesis by induced mutations in the tumour-suppressor gene in the normal skin cells leading to a transformation of normal cell into cancerous cells by halting the initiation of programmed cell death. An example of this mutation is seen in the alteration of a guanine in the p53 gene through oxidative mechanisms in basal cell and squamous cell carcinoma [43].

DNA damage is pivotal in cancer formation. A study proposed that FPP was capable of impeding DNA fragmentation induced by free radicals and H_2O_2-induced DNA damage at a dose of 100 µg/mL [16]. In addition, the aqueous extract of *Carica papaya* fruit suppressed proliferation of human breast epithelial cancer (MCF-7) cells in vitro. The aqueous extract of *Carica papaya* showed significant anti-proliferation activity (~53%) in MCF-7 cells at a dose of 4% *v*/*v* after 72 h treatment. The anti-cancer activity of FPP might be attributed to the mechanisms including triggering cell signaling to induce apoptosis [78]. An in vitro study showed that the aqueous extract of *Carica papaya* leaves showed antiproliferation activity of MCF-7 at a IC_{50} of 1.31 mg/mL and induced apoptosis of MCF-7 cells at 22.5% with a dose of 0.65 mg/mL [79].

Carica papaya enriched with phytochemicals, including flavonoids, has been found to possess chemopreventive properties. The mechanisms of action underlying the chemoprevention effects include activating tumour-suppressor genes, deactivating oncogene products transcriptionally, decreasing oxidative damage via acting as free radical scavengers and impeding the commencement of lipoxygenase reaction by chelating with ROS-generating agents. For example, the benzene fraction of aqueous extract of *Carica papaya* showed chemoprotective effects in benzo(a)pyrene and 7,12-dimethyl benz(a)anthracene -induced carcinogenic animal models. It was reported that a significant reduction of lung adenomas (>50%) at a treatment dose of 1 g/kg body weight. In addition, a significant reduction in skin papillomagenesis incidence at 64.20% was compared with tumour incidence in the control group. It was suggested that those flavonoids contained within different parts of the *Carica papaya* plant act via multi-signaling networks as the viable chemoprevention agents [80].

An in vivo study reported that FPP at a concentration of 500 mg/kg significantly elevated antioxidant enzymes, including GPx (66.1%), SOD (20%), and CAT (81%). Furthermore, FPP was also capable of preventing DNA structural damage possibly induced by free radicals and genotoxins [81]. This was supported by another study suggesting that *Carica papaya* peel extract significantly increased glutathione (GSH), while decreasing MDA and ROS production. Thus, preventing DNA damage and induction of colonic carcinogenesis azoxymethane treated group [82]. Another study by Mukami and colleagues revealed that orally administration of FPP at 450 mg/kg showed complete disappearance of the tumours in a radiation-induced leukemia mice model [83]. Overall, several research groups revealed the anticancer properties of papaya extracts. Further studies are needed to standardize the extract for quality control of the efficacy, and discover novel compounds, owing to the anticancer activities.

4. Conclusions

To summarize, the *Carica papaya* counteracts oxidative stress via its potent antioxidant properties. Therefore, it can be incorporated into nutraceuticals or conventional medications to be used as a potential preventative or treatment option for various health

conditions. The antioxidant properties of the *Carica papaya* plant might be attributed to the various chemical constituents that the plant contains, including caffeic acid, myricetin, quercetin, rutin, α-tocopherol, papain, BiTC, kaempferol steroids, alkaloids, and saponins.

There is no doubt that emerging evidence has proven the potential of *Carica papaya* as a natural resource that can be exploited as a medicinal product. However, more safety data are needed to justify its use in different medical conditions. Many plants—although exerting therapeutic benefits, having been used traditionally for diseases since the ancient times—are potentially cytotoxic [7,84]. The acute toxicity study of the *Carica papaya* leaf extract revealed that there were no significant toxic effects of *Carica papaya* leaf extract at the concentration up to 2 g/kg of body weight, which corresponded to 14 times the dose incorporated in traditional medication. Moreover, it was also suggested that any concentration of *Carica papaya* leaf extract below 2 g/kg of body weight posed no significant toxicity and adverse effects when administered orally for a 14-day interval [85,86]. In terms of the *Carica papaya* extract with different methods of extraction, namely ethanol and aqueous extract, it might possess different safety profiles, owing to the extractive chemical constituents. For example, the ethanol extract might be more nephrotoxic and hepatotoxic than the aqueous extract in the Wistar rats model at 1 g/kg of body weight concentration [87]. However, all these studies suggest that more extensive evaluation studies pertaining the cytotoxicity profile of oral administration of the *Carica papaya* extract are needed to further validate the safety for consumption.

It was also suggested that the medicinal properties of the *Carica papaya* plant can be attributed to other mechanisms of action. Several studies have suggested that *Carica papaya* extract exerted antimicrobial properties that aided in wound recovery [69,71]. Therefore, more studies should be done in order to unravel the benefits of the *Carica papaya*.

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Curcuma amarissima Extract Activates Growth and Survival Signal Transduction Networks to Stimulate Proliferation of Human Keratinocyte

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Simple Summary: Like many plants in the family of Zingiberaceae, *Curcuma amarissima* has been traditionally used to induce healing and tissue regeneration. However, there is no scientific evidence to explain how *Curcuma amarissima* works to accelerate wound healing. Our data clearly proved that *Curcuma amarissima* extract could potentially accelerate the closure of scratch wounds of human keratinocytes by stimulating cell proliferation. The potential mechanisms underlying these effects were defined to be associated with the activated signal transduction pathways relevant to cell proliferation and survival. This strongly suggests the ability of *Curcuma amarissima* to enhance the process of keratinocyte reepithelization during wound healing. Our current study provides convincing evidence that supports the possibility to develop an effective wound-healing promoting agent from this plant.

Abstract: Many medicinal plants have been used to treat wounds. Here, we revealed the potential wound healing effects of *Curcuma amarissima* (CA). Our cell viability assay showed that CA extract increased the viability of HaCaT cells that were cultured in the absence of serum. This increase in cell viability was proved to be associated with the pharmacological activities of CA extract in inducing cell proliferation. To further define possible molecular mechanisms of action, we performed Western blot analysis and immunofluorescence study, and our data demonstrated that CA extract rapidly induced ERK1/2 and Akt activation. Consistently, CA extract accelerated cell migration, resulting in rapid healing of wounded human keratinocyte monolayer. Specifically, the CA-induced increase of cell monolayer wound healing was blocked by the MEK inhibitor (U0126) or the PI3K inhibitor (LY294002). Moreover, CA extract induced the expression of Mcl-1, which is an anti-apoptotic protein, supporting that CA extract enhances human keratinocyte survival. Taken together, our study provided convincing evidence that *Curcuma amarissima* can promote proliferation and survival of human keratinocyte through stimulating the MAPK and PI3K/Akt signaling cascades. These promising data emphasize the possibility to develop this plant as a wound healing agent for the potential application in regenerative medicine.

Keywords: human epidermal keratinocytes; HaCaT; *Curcuma amarissima*; PI3K/AKT; RAS/ERK; wound healing; proliferation; survival

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1. Introduction

Ineffective skin wound healing is becoming a big problem in the public health sector. Several factors including aging, diabetes, infection, immunodeficiency, and cancers can lead to unsuccessful wound treatment and eventually cause morbidity and mortality [1–5]. Although the human body has a great protective skin barrier, sometimes an unexpected injury is unavoidable. A process of organisms, namely "wound healing", is responsible for the reconstruction of injured skin. This physiological repairing system requires four stages, which include hemostasis (blood clotting), inflammation, proliferation (growth of new tissue), and maturation (remodeling) [6,7]. A successful step of re-epithelialization is considered to be an essential indicator of wound closure to prevent further infection and chronic wound development [8]. In response to skin damage, the process called re-epithelialization is triggered to restore the damaged epidermis. The most important cell types responsible for re-epithelialization are keratinocytes, which proliferate, differentiate, and migrate to heal the open wound [9]. Failure of keratinocytes to maintain skin integrity and/or wound closure could cause reoccurrence of affected lesions and further complicated skin reaction [10]. At the cellular and molecular level, growth and survival signaling cascades are very crucial for enhancing certain stages of re-epithelialization. Specifically, the signaling pathway of mitogen-activated protein kinase (MAPK) is involved in regulating cell proliferation and migration [11,12]. In particular, ERK1/2 kinase phosphorylation is induced by various groups of growth factors [13–16]. Therefore, certain growth factors such as EGF or PDGF are approved to be used to stimulate the skin healing process [17–19]. Nevertheless, growth factors (used at high concentrations) can cause adverse effects, including uncontrolled cell growth, chronic inflammatory skin disease such as psoriasis, or aberrant skin functions [20,21]. Therefore, discovering novel wound healing agents that have lesser side effects would be beneficial for patients with certain conditions.

Since ancient times, people have used herbal medicines as one of the components in wound management to accelerate wound healing. In particular, for treatments derived from plants, both topical and systemic herbal agents have been widely used in wound healing. Several properties, including anti-inflammatory, antioxidant, and antimicrobial activities, are required for being effective wound healing agents [22,23]. Thus, any herbs with these properties will be worth investigating and using for the development of effective wound healing agents. Generally, extracts or the isolated phytochemical compounds derived from plants promote the tissue regeneration process via certain mechanisms, which ultimately provide a synergistic effect on healing efficiency [24]. Currently, some natural products that include herbs [25] have been included as active components for wound treatments. For instance, it has been demonstrated that *Poincianella pluviosa* extract could potently accelerate keratinocyte migration and proliferation [26]. Recently, *Boesenbergia rotunda* or fingerroot, belonging to the Zingiberaceae family (same family with *Curcuma amarissima*) has been revealed to promote human keratinocyte proliferation via stimulating the phosphorylation of ERK1/2 and Akt [27]. Although many plant-derived medicines are believed to be effective, affordable, and cause minimal unwanted side effects [28], it is necessary to carefully define the exact mechanisms of action to be certain how medicinal plants function.

Curcuma amarissima Roscoe (CA) or "black turmeric" or "Kamin-dum" is in the family Zingiberaceae and is usually used to treat amoebic dysentery, enteritis, and vermicide [14]. Lectin isolated from the rhizomes of this plant showed anti-fungal activity against several different species, including *Colectrotricum cassiicola, Exserohilum turicicum,* and *Fusarium oxysporum*. Moreover, the purified lectin could also reduce cell proliferation in a breast cancer cell line, BT474 [29]. Accumulating evidence has suggested that plants in the Zingiberaceae family may be one of the great candidates for wound healing [27,30,31]. Specifically, several activities of herbs in this family contain essential combinations that are necessary for the healing process. Those activities include antioxidant, antimicrobial, anti-inflammation, promoting collagen production, promoting dermal cell proliferation, promoting new blood vessels, and central/peripheral antinociceptive effects [32,33]. Previ-

ous studies disclosed that the rhizomes of CA contain several different active constituents similar to those found in *Curcuma longa*. Those compounds include curcumenol, curdione, curzerenone, germacene, isofrtungermacrene, and zedoarone [34].

Although many plants in the family of Zingiberaceae have been revealed to have promising effects suitable for wound treatment, there is no evidence showing that *Curcuma amarissima* Roscoe has regenerative effects on skin. Therefore, it is of our interest to investigate whether the *Curcuma amarissima* Roscoe extract has specific pharmacological activities that help enhance wound healing processes.

Here, we discovered that CA can enhance human keratinocyte, HaCaT, cell proliferation and migration via inducing ERK1/2, and Akt phosphorylation, which are important molecular pathways involved in re-epithelialization. Our current study provided information that CA can be developed as an agent for accelerating skin wound repair.

2. Materials and Methods

2.1. Preparation of Ethanolic Extract from the Rhizomes of Curcuma aeruginosa (CA)

The rhizomes of *Curcuma amarissima* Roscoe were obtained from the cultivating areas in Mae Taeng District, Chiang Mai, Thailand, and were identified by a botanist at the Faculty of Pharmacy, Chiang Mai University. The samples of authenticated *Curcuma amarissima* Roscoe were deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, with the voucher specimen number 0023261. For preparing the ethanolic extract, the fresh rhizomes of *Curcuma amarissima* Roscoe were washed, cut into small pieces, dried, and ground. Next, the ground powder was mixed with ethanol (95%) at room temperature (RT) for 24 h. The mixture was filtered through Whatman No.1 filter paper (Sigma-Aldrich, Saint Louis, MO, USA), and the filtered solution was subjected to a rotary evaporator at 40 °C to eliminate the solvent. Next, one gram (g) of the obtained CA extract was diluted in 1 milliliter (mL) 100% dimethyl sulfoxide (DMSO) and used as a stock solution. For each treatment, the CA extract stock solution (1 g/mL in DMSO) was further pre-diluted in medium to obtain the final working concentrations. However, the final concentration of DMSO was not allowed to exceed 0.5% v/v in the diluted media throughout the experiment.

2.2. HPLC Fingerprint of CA Extract

HP1100 system (HPLC LC-10, Shimadzu, Kyoto, Japan) with an Agilent C-18 column (150 × 4.6 mm, 5 μm) was applied for visualizing the HPLC fingerprint of the extract. The system was performed with a thermostatically controlled column oven and a UV detector set at 254 and 360 nm. The mobile phase was methanol–water system with gradient elution as follows: 40–70% methanol for 0–30 min, 100% methanol for 80–100 min, 40% methanol for 105–115 min. The extract was diluted with methanol to 50 mg/mL before injection (10 μL of sample volume) into the column, with 1.0 mL/min of flow rate.

Additionally, quantitative analysis of curcuminoids and polyphenolic contents in CA extract by HPLC was performed. CA extract was determined for the existence of curcuminoid and polyphenolic contents by HPLC using a C18 column (250 × 4.6 mm, 5 μm) (Agilent Technologies, Santa Clara, CA, USA). The detection of curcuminoids, including bis-demethoxycurcumin, demethoxycurcumin, and curcumin, was carried out using isocratic mode of mobile phase (2% acetic acid in water and acetonitrile 50:50 v/v) with a flow rate of 1 mL/min. Ten microliters of the extract (20 mg/mL dissolved in 1 mL of MeOH) was injected into the column, and detection was done at 425 nm. The polyphenolic content in the extract was determined by using a gradient system of mobile phase A (1% acetic acid in water) and mobile phase B (100% acetonitrile) with a total run time of 50 min for the detection with a flow rate of 0.7 mL/min and detection wavelength at 280 nm. The gradient system used was 90% A in 0 min–60% in 28 min, followed by 40% in the next 39 min and 10% in the next 50 min.

2.3. Culture of Human Keratinocyte Cell Line, HaCaT

HaCaT cell line was purchased from CLS Cell Lines Service GmbH (CLS Cell Lines Service GmbH, Eppelheim, Baden-Wurttemberg, Germany) and maintained in DMEM (Gibco, New York, NY, USA), supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) (Gibco, New York, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin) (Gibco, New York, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2. For experiments that required serum deprivation, cells were cultured in serum-free media.

2.4. Cell Viability Determination of HaCaT Cells Treated with CA Extract

MTT assay was conducted in CA-extract-treated HaCaT cells, which were cultured in either media containing fetal bovine serum (FBS) or FBS-free media. The viability characteristics of the cell were observed. Moreover, toxic and non-toxic concentrations of CA were obtained. Briefly, we cultured (5×10^4 cells/well in 96-well plates) in complete media overnight. For CA extract treatment, the CA extract stock (1 g/mL) was pre-diluted in FBS-free media to make a final concentration of 160 µg/mL (containing DMSO at 0.16%). DMSO (as a vehicle control) was diluted in the same manner. Next, we prepared the treating media containing various concentration of CA extract (ranging from 160 down to 0.625 µg/mL) and DMSO (0.000625–0.16%) by making a two-fold dilution of CA extract or DMSO in either FBS-rich or FBS-free media. Cells were treated with individual treating media (200 µL/well in 96-well plates) for 48 h and subjected to a working solution (0.4 mg/mL) of MTT reagent for 1 h in an incubator. After MTT was discarded, 100 µL of DMSO was added to each well, and the developed color was detected (at 570 nm) by an absorbance reader plate spectrophotometer (BioTek Instruments, Winooski, VT, USA). From this experiment, we chose three concentrations (2.5, 5, and 10 µg/mL) of CA extract for all experiments. Moreover, the proliferative effect of CA extract at specific non-toxic concentrations on HaCaT cells was determined by phase-contrast microscopy. For this experiment, cells were treated with CA extract (2.5, 5, and 10 µg/mL) in FBS-rich media or FBS-free media, and the changes in the size of cell colonies were captured at various time points (0, 24, 48, and 72 h). In some experiments, HaCaT cells were fixed and permeabilized with absolute methanol and stained with crystal violet to clearly verify the density and the size of the colonies.

2.5. Determination of HaCaT Cell Monolayer Healing by Scratch Wounding Assay

Wound healing assay of HaCaT cell monolayer was performed. Briefly, confluent HaCaT cells cultured in 24-well plate were scratched by a P200 pipette tip to make a thin wound. Scratch wounds were created in confluent monolayers using a P200 disposable micropipette tip. Cells were then treated with CA extract at all three non-toxic concentrations diluted in serum-deprived media, with or without 10 µM U0126 (CST, Boston, MA, USA) or 50 µM of LY294002 (CST, Boston, MA, USA). The rate of keratinocyte migration was monitored over time, and representative pictures were taken at different time points (0, 20, 30, 40, and 50 h). The measured wound areas were analyzed by the ImageJ program.

2.6. Effects of CA Extract on Increasing Number of HaCaT Cells

We directly counted total number of CA extract-treated cells to confirm the effects of CA extract on inducing keratinocyte proliferation. Low density of cells (2.5×10^4 cells/well) was seeded in 24-well plates overnight. Then, media were changed to serum-free media before treating cells with 10 µg/mL. At each incubation time point (0, 24, and 48 h), cells were detached from the plate and counted. The differences in the number of cells between control and experimental groups were calculated.

2.7. Effects of CA Extract on Stimulating Molecular Signaling Pathways

To investigate the responsible molecular signaling in which CA extract can be activated, Western blot analysis was conducted to detect certain molecular players responsible for conveying signal transduction for cell proliferation and survival. Specifically, cells were

seeded in complete media in 3-cm dishes for 24 h. Cells were then cultured in media without serum for 24 h. For a positive control, HaCaT cells were treated with 100 ng/mL of EGF for 15 min before harvesting. For determining whether CA extract stimulates the early signaling, we treated HaCaT cells with 10 μg/mL of CA extract and harvested them at a certain time (0 min to 24 h). For evaluating the concentration-dependent effects of CA extract, we treated HaCaT cells with 3 different non-toxic concentrations of CA extract for 15 min (with or without U0126 or LY294002). For any experiment involving inhibitors, cells were pre-treated with inhibitors (10 μM of U0126 or 50 μM of LY294002) for 2 h prior to CA extract addition. For the experiment that aimed to detect the level of Mcl-1 protein, cells were treated with CA extract at 2.5, 5, 10 μg/mL, or DMSO as a vehicle control, for 24 h before harvesting cells. The effects of CA on inducing the expression and activating the phosphorylation of ERK1/2 (pERK1/2), and Akt (pAkt), cells were treated with CA extract (10 μg/mL) for 30 min. After preparing cell lysates, all samples were subjected to SDS-PAGE and Western blot analysis. Membranes were then incubated with 5% skim milk dissolved in TBST (Sigma-Aldrich, Saint Louis, MO, USA) at room temperature (RT) for 1 h. After washing trice, membranes were incubated with primary antibodies for 24 h at 4 °C. Primary antibodies (Cell Signaling Technology (CST), Boston, MA, USA) included (1) an anti-pErk1/2 antibody, (2) an anti-total Erk1/2 antibody, (3) an anti-pP38 antibody, (4) an anti-total p38 antibody, (5) an anti-pSAPK/JNK antibody, (6) an anti-total APK/JNK antibody, (7) an anti-pAkt antibody, (8) an anti-total Akt antibody, (9) an anti-pPRAS40 antibody, (10) an anti-pEGFR antibody, (11) an anti-total EGFR antibody, (12) an anti-Mcl-1 antibody, and a an anti-β-actin antibody. Next, membranes were incubated with appropriate secondary antibodies (Li-COR Biosciences, Lincoln, NE, USA), which were an anti-mouse IgG (conjugated with IRDye®800CW) or an anti-rabbit IgG (conjugated with IRDye®680RT) for 2 h, at RT. The Western blot signal was detected with Odyssey® CLx Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and the intensity of each immunoreactive band was analyzed by Image J.

2.8. Detection of Early Signaling Pathway Induced by CA Extract in Individual Cells by Immunofluorescence Study

The phosphorylation of key kinases including ERK1/2 (pERK1/2), Akt (pAkt), EGFR (pEGFR at tyrosine 1068), and the expression of total EGFR and Mcl-1 proteins upon CA extract stimulation were determined in individual cells by immunostaining technique. Sample cover slips were prepared by seeding HaCaT cells on glass cover slips to confluence, and cells were then treated with CA extract (10 μg/mL) for 30 min. Next, fixation was performed, 15 min at RT, using 4% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO, USA). After washing three times with PBS, 0.3% Triton X-100 was added to permeabilize cells for 5 min. Sample cover slips were then blocked with 1% BSA for 1 h at RT and incubated with primary antibodies overnight at 4 °C. Primary antibodies were an anti-pErk1/2 antibody, an anti-pAkt antibody, an anti-pEGFR receptor antibody, an anti-total EGFR antibody, and an anti-Mcl-1 antibody. Sample coverslips were then probed with anti-rabbit IgG (Alexa488-conjugated) or goat anti-rabbit IgG (Alexa594-conjugated) (Thermo Fisher Scientific, Waltham (HQ), MA, USA) for 2 h at RT. The nuclei of HaCaT cells were stained with DAPI (1 μg/mL) (Sigma-Aldrich, Saint Louis, MO, USA). Sample coverslips were washed three times with PBS, and one time with distilled water before being subjected to mounting with Fluoromount-G (SouthernBiotech, Bermingham, AL, USA) as a mounting medium. Positive fluorescent signals were detected and recorded by a fluorescence microscope, Axio Vert.A1 (Carl Zeiss Suzhou Co. Ltd., Suzhou, China) equipped with Colibri 7 illumination system (Carl Zeiss Microscopy GmbH, Gottingen, Germany) and Axiocam 506 color digital camera (Carl Zeiss Microscopy GmbH, Gottingen, Germany) using immersion objective (100×/1.3 Oil M27). Signal of the nuclei stained with DAPI was excited by the UV mode (385/30 nm), signal of Alexa488 was excited by the blue light mode (469/38 nm), and signal of Alexa594 was excited by the green mode (555/30 nm). Representative pictures were taken with the Zen 2.6 (blue edition) Software.

2.9. Data and Statistical Analysis

Results were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed, followed by Tukey's post hoc multiple comparisons (SPSS Inc., Chicago, IL, USA). *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Curcuma Amarissima (CA) Extract Enhances Cell Viability of HaCaT Cells

After obtaining the extract, we performed chromatographic fingerprint analysis of the ethanolic extract from *Curcuma amarissima* (CA) by high-performance liquid chromatography (HPLC). The results (both detected at 254 and 360 nm) showed the unique characteristics of compound fingerprint in CA extract (Figure S1). Like many plants in the genus *Curcuma* such as *Curcuma longa*, *Curcuma amarissima* may contain some active compounds including curcumin that has been shown to have significant wound healing properties. Therefore, quantitative analysis of curcuminoids in *Curcuma amarissima* extract was examined by HPLC. As expected, the results revealed the existence of two curcuminoids in the extract which were desmethoxycurcumin (5.87 ± 0.001 μg/g extract) and curcumin (10.81 ± 0.001 μg/g extract) (Figure 1). We also found that the extract contained ferulic acid (Figure S2).

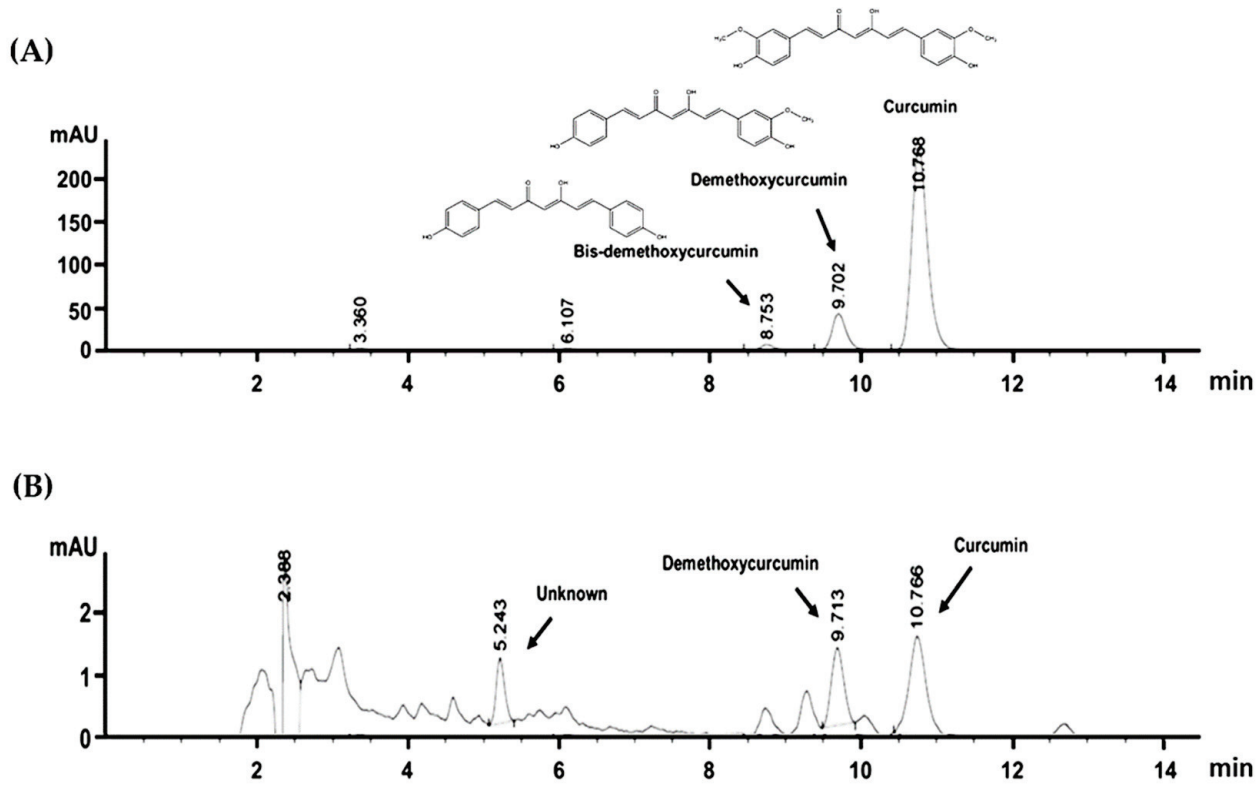

Figure 1. The HPLC profile of (**A**) curcuminoids (50 μg/mL) including bis-demethoxycurcumin, desmethoxycurcumin, and curcumin with their chemical structures. (**B**) The HPLC profile of *Curcuma amarissima* extract (20 mg/mL) indicating the existence of desmethoxycurcumin and curcumin.

We performed MTT cell viability assay to monitor changes in cellular metabolic activity and viability of HaCaT cells in media containing CA extract (CA extract in DMSO as a solvent from the stock solution) with the presence of 10% Fetal Bovine Serum, FBS. Cells were also treated with the FBS-rich media containing DMSO (as a vehicle control) with varied concentrations corresponding to those present in the CA extract-treated group. Results demonstrated that CA extract at concentrations lower than 40 μg/mL had no significant effect on the viability of HaCaT cells cultured in FBS-rich media (Figure 2A).

However, the extract at 80 and 160 μg/mL caused significant reduction in cell viability to approximately 30% and 10%, respectively. However, DMSO at all concentrations used did not cause any change in HaCaT cell viability (Figure 2A). We also performed similar experiments where treatment with CA extract was done in FBS-free media. Results showed that HaCaT cell viability in serum-free media was more sensitive to CA treatment. In particular, when cells were treated with CA at 20 μg/mL, cell viability was observed to be significantly reduced, and the cell viability was maximally decreased to approximately 20% in cells treated with CA at 40 μg/mL or more (Figure 2B). Interestingly, we observed that HaCaT cell metabolic activity dramatically increased in response to treatment with CA extract at 2.5, 5, and 10 μg/mL (Figure 2B). The results indicate that CA extract induced HaCaT cell metabolic activity, and this activity may be caused by an increasing number of cells upon CA extract treatment.

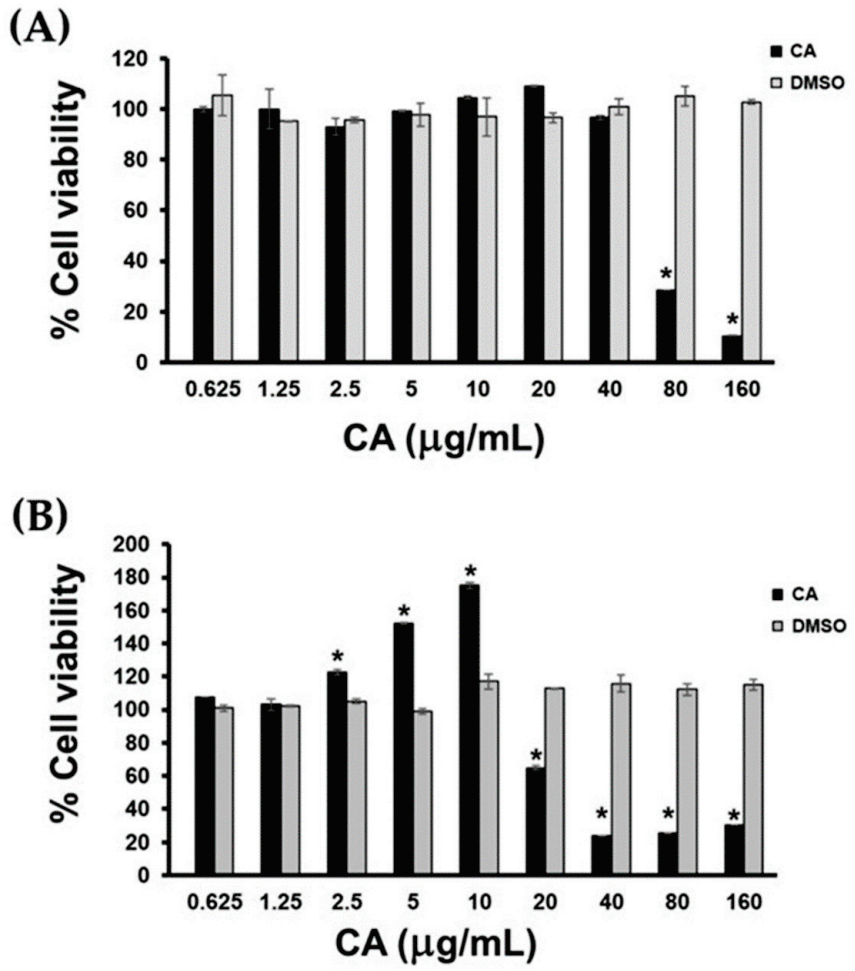

Figure 2. Cell viability HaCaT cells upon treatment with *Curcuma amarissima* (CA) extract. Viability of HaCaT cells treated with CA extract (0.625 to 160 μg/mL) or DMSO (0.000625–0.16%) for 48 h in FBS-rich media (**A**) and in FBS-free media (**B**). Data from three experiments were analyzed and presented as mean ± standard deviation (SD); * $p < 0.05$ in comparison to the untreated group.

3.2. CA Extract Stimulates Colony Formation and Proliferation of HaCaT Cells

From the previous experiment, data clearly showed that CA extract at non-toxic concentrations (2.5, 5, and 10 μg/mL) could significantly induce HaCaT cell metabolic activity in a concentration-dependent fashion, which could be seen when serum was deprived. We thought that an increase in cell viability of HaCaT cells may have resulted from the proliferative effects of CA extract. Therefore, we observed whether CA extract can accelerate the growth of the HaCaT colonies over time by a phase-contrast microscope. Our

data demonstrated that at 0 h of CA treatment or DMSO vehicle control (in both complete and serum-free media), cells were detected to be single cells with equal distribution and similar cell density on the surface of the dish (Figure 3A,B). Over the course of 72 h, we noticed that the colony size of HaCaT cells incubated with CA extract in FBS-rich media gradually increased from single cells at 0 h to 100% confluence at 72 h (Figure 3A). We also observed that the colonies of CA-extract-treated cells (at 10 μg/mL) were slightly larger than those of the untreated or DMSO-treated cells (Figure 3A). These observations were clearly verified by the results obtained from CA treatment in serum-free media, where HaCaT cells were suppressed to undergo slow cell proliferation due to the lack of growth factors in FBS that generally stimulate cell proliferation. Specifically, CA extract at 10 μg/mL showed strong effects on accelerating the growth of the HaCaT colonies at 24, 48, and 72 h of incubation (Figure 3B).

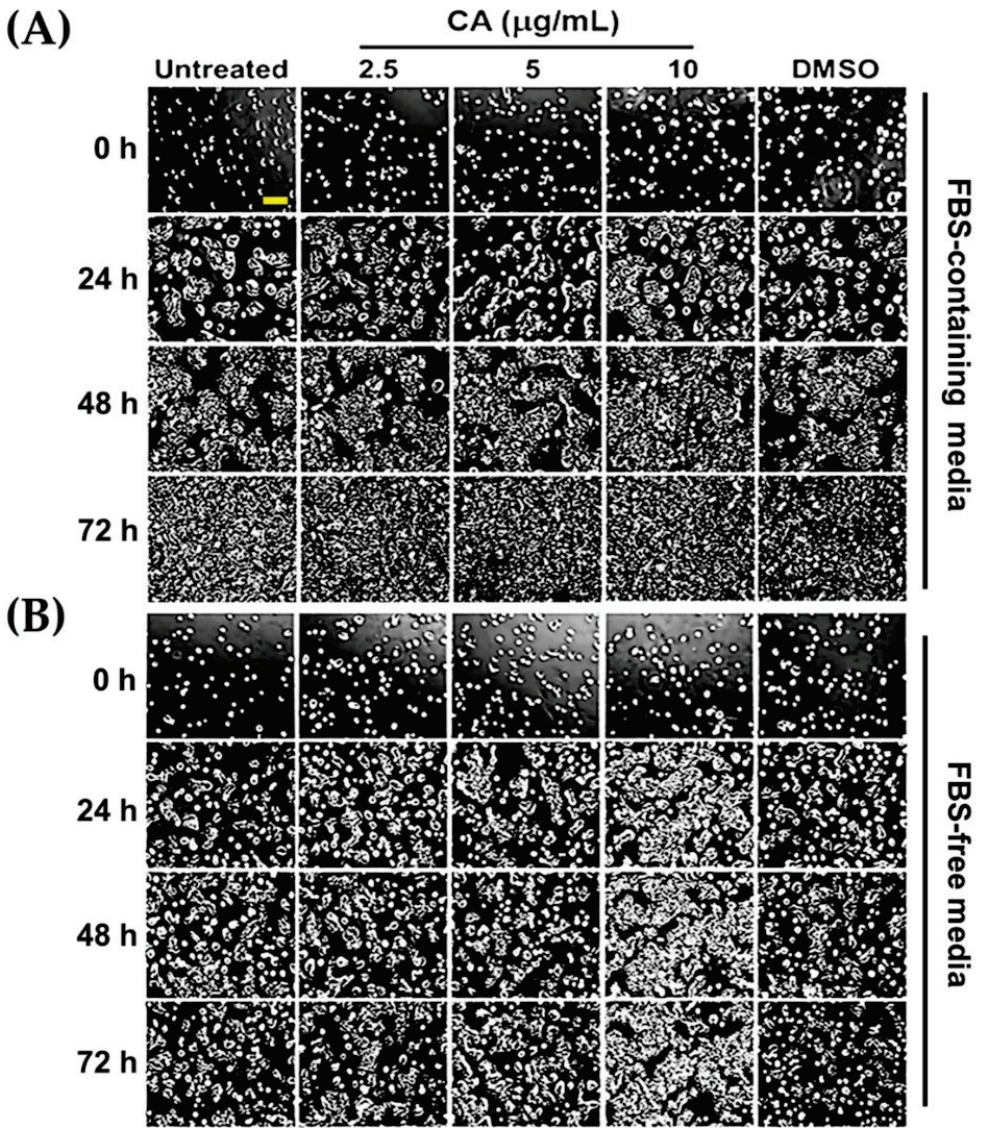

Figure 3. CA extract induced the growth of HaCaT colonies. HaCaT cells were left untreated or treated with CA extract at 2.5, 5, and 10 g/mL in media containing 10% FBS (**A**) or in media without the presence of FBS (**B**). The sizes of HaCaT colonies were monitored, and the pictures were captured at 0, 24, 48, and 72 h by a phase-contrast microscope (10× magnification, scale bar = 200 μm). Cells were also treated with DMSO which served as a vehicle control. The pictures were representative of three individual experiments.

We attempted to clarify whether the observed phenomenon is an effect of CA extract on stimulating cell proliferation. Therefore, we treated HaCaT cells with CA extract (5 and 10 μg/mL) in media containing FBS or FBS-free media for 2 days, and then cells were stained with crystal violet and we observed clear phenotypic changes of the HaCaT colonies (Figure 4A). Consistent with these results, our cell counting assay revealed that CA extract exhibited the ability to stimulate an increase in cell number over 48 h (Figure 4B).

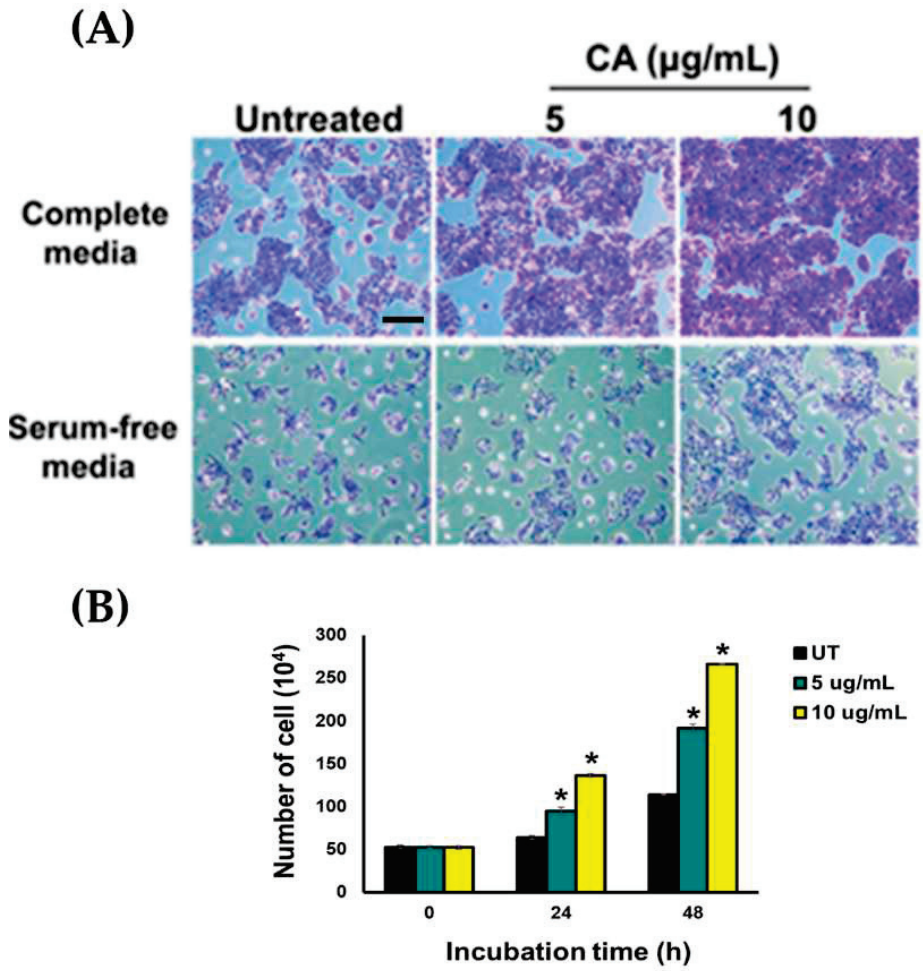

Figure 4. (**A**) Crystal violet staining of CA-extract-treated cells (5 and 10 μg/mL) for 24 h in FBS-rich media or FBS-free media detected by a phase-contrast microscope (10× magnification, scale bar = 200 μm). (**B**) Analysis of total number of cells treated with CA extract at 5 and 10 μg/mL over the course of 24 and 48 h in FBS-free media. Data, from three experiments, present mean ± SD; * $p < 0.05$ (in comparison to the untreated (UT) group).

3.3. CA Extract Induces Migration of HaCaT Cell Monolayer into The Wounded Area

Besides proliferation, migration of human keratinocytes is an additional crucial step that contributes to efficient healing. To test that CA extract can stimulate HaCaT cells migration, we monitored the rate of migration into the wounded area of keratinocytes treated with CA extract at varied concentrations and time points. Our quantitative analysis clearly demonstrated that CA extract significantly promoted percent cell migration into the wounded areas in a concentration-dependent manner and in all time points (20, 30, 40, and 50 h), compared to those of the untreated and DMSO-treated groups (Figure 5A,B).

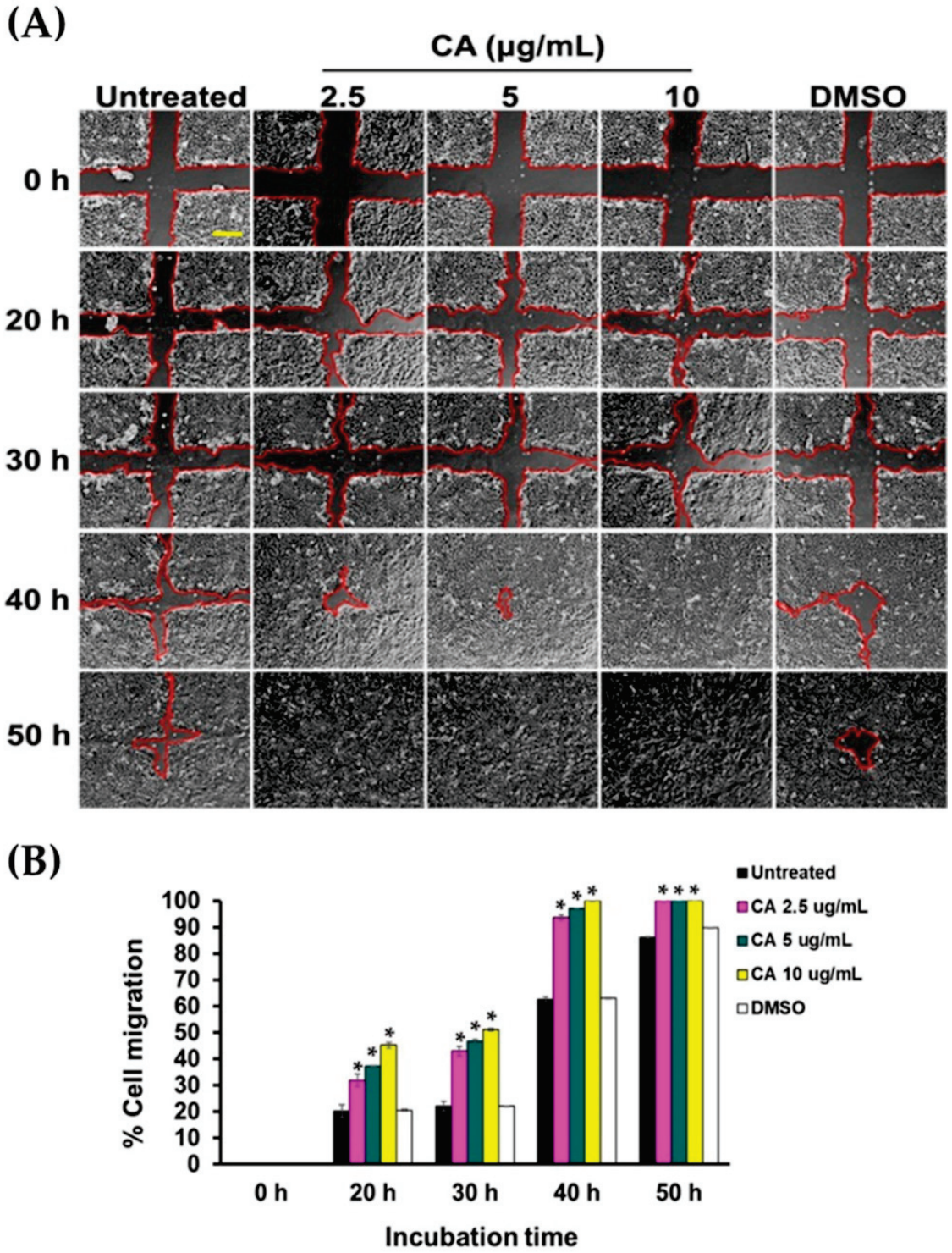

Figure 5. CA extract stimulated HaCaT cell monolayer healing. (**A**) HaCaT cell monolayer treated with CA extract at various concentrations in FBS-free media was monitored for the ability to heal over the course of 50 h by a phase-contrast microscope (10× magnification, scale bar = 200 μm). The vehicle control group was treated with DMSO. (**B**) The analysis of percent migration of CA extract-treated HaCaT cells in FBS-free media at each time point (0, 20, 30, 40, or 50 h). Regions confined by the red lines indicate the space with no cell occupation. Data present mean ± SD (* $p < 0.05$, compared to the untreated).

3.4. Proliferation and Survival Signal Transductions Are Induced in HaCaT Cells upon Treatment with CA Extract

Since we observed that CA induced HaCaT cell proliferation and migration, we hypothesized that CA extract stimulates growth and survival signaling in HaCaT cells. Therefore, we tested our hypothesis by focusing on relevant molecular signaling cascades. It is well-defined that the MAPK signaling pathway is important for activating cell proliferation and migration. In particular, this signal transduction pathway is active in response to damage of epidermis, and blockade of ERK activation suppresses keratinocyte migration. Therefore, we investigated the effects of CA extract on stimulating ERK phosphorylation by Western blot analysis. Our data (Figure 6A) revealed that when compared to the untreated group, CA extract rapidly stimulated ERK1/2 phosphorylation (pERK1/2) approximately 3 fold, starting at 5 min after treatment and reaching the maximum activation at 1 h post-treatment. Phosphorylation of ERK1/2 was determined to be approximately 10 fold (Figure 6B). The phosphorylation of ERK1/2 upon CA extract treatment exhibited the unique pattern of a bell-shaped curve where ERK1/2 phosphorylation gradually increased over time and then decreased after 1 h of CA stimulation (Figure 6A,B). Observing ERK1/2 activation led us to the belief that CA may also stimulate the survival signal transduction pathway. Therefore, we detected the activation of Akt by examining phosphorylation of serine 473 of Akt, which is normally responsible for promoting cell survival by inhibiting apoptosis and regulating cell cycle. Data from Western blot analysis showed that CA could rapidly activate Akt phosphorylation, and the phosphorylation pattern was similar to that of pERK1/2. Consistently, PRAS40 which is a downstream substrate of active Akt was also phosphorylated, and the maximal phosphorylation was detected to be between 1 to 3 h post-CA extract treatment (Figure 6A). The rapid activation of ERK1/2 and Akt by CA extract may have occurred as a result of the activation of the upstream molecules of the signaling cascade. Therefore, we detected the activation of epidermal growth factor receptor (EGFR) by targeting tyrosine 1068 (Y1068) phosphorylation and found that CA extract did not activate EGFR receptor, in comparison to the results obtained from HaCaT cells activated with EGF, where EFGR was strongly phosphorylated (Figure 6A).

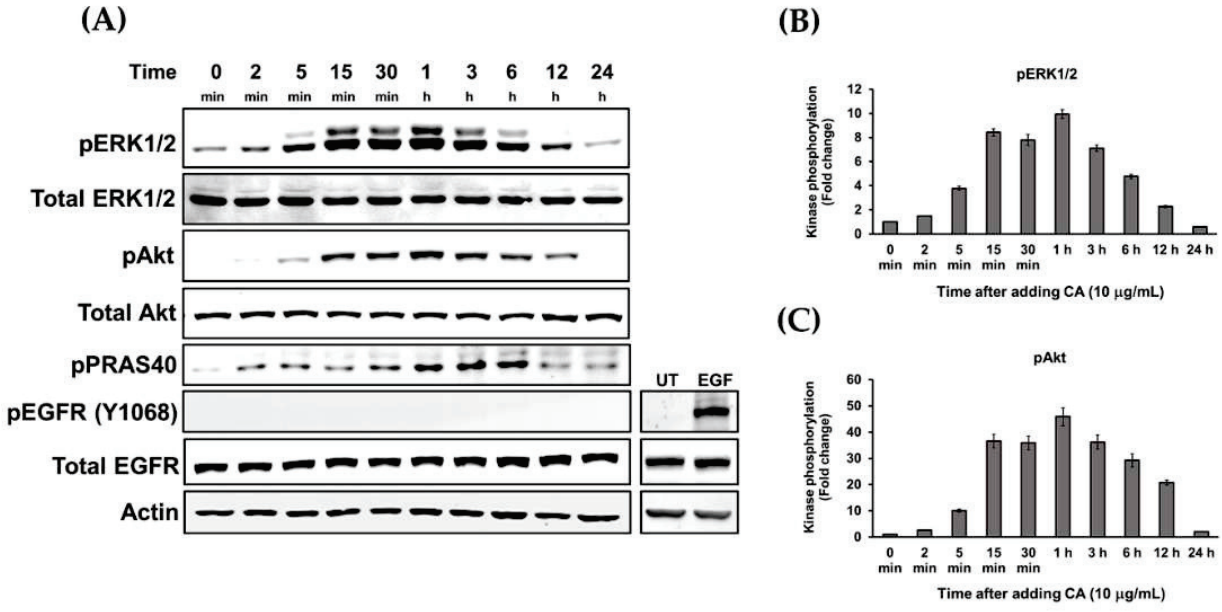

Figure 6. CA extract strongly stimulated ERK1/2 and Akt phosphorylation. (**A**) Time-dependent detection by Western blot analysis for the expression and phosphorylation of crucial signaling molecules (ERK1/2, Akt, PRAS40, and EGFR) in HaCaT cells treated with 10 μg/mL of CA extract over 24 h. (**B**) Quantification of the phosphorylation of ERK1/2 in CA extract-incubated HaCaT cells. (**C**) Quantification of the phosphorylation of Akt in CA extract-treated HaCaT cells. Actin was used as a loading control. Data from three individual experiments present mean ± SD; $p < 0.05$.

Besides Western blot analysis, an immunofluorescence study was done to clearly verify that CA extract activates ERK1/2 and Akt in individual cells. Our studies confirmed that CA extract at 10 μg/mL could strongly induce phosphorylation of ERK1/2 (Figure 7A) and Akt (Figure 7B) in human keratinocytes, but this activation was not seen in the untreated group. Nevertheless, CA extract had no effect on inducing EGFR phosphorylation (Figure 7C) or the receptor's expression pattern (Figure 7D).

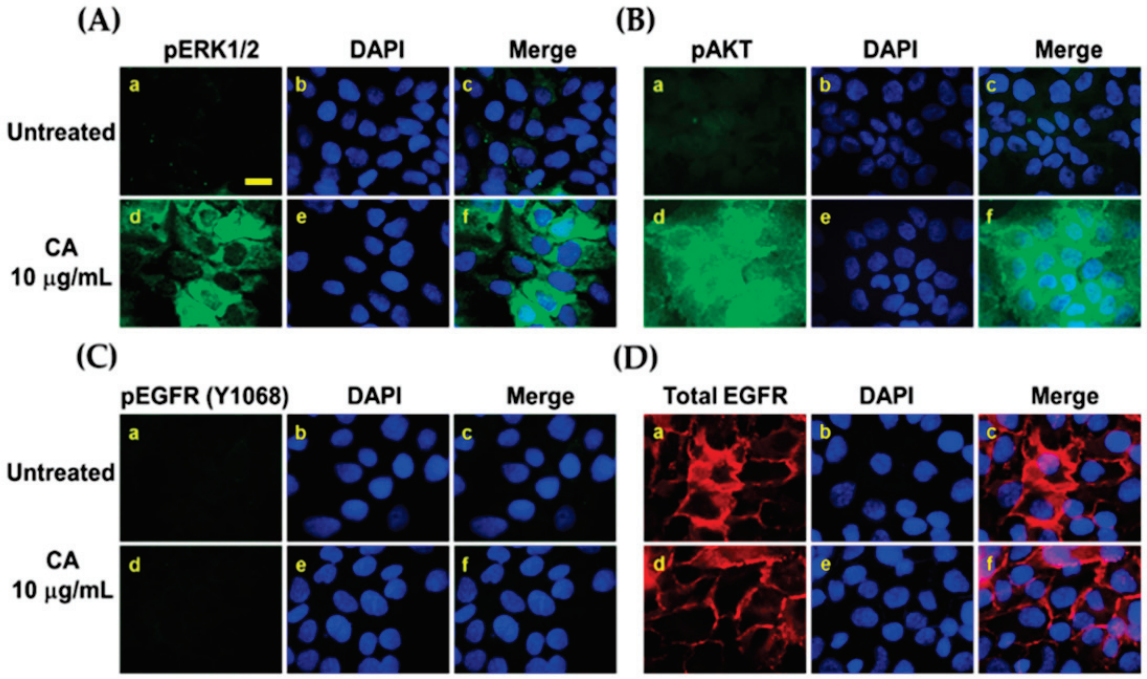

Figure 7. CA extract activated ERK1/2 and Akt phosphorylation in single cells. Immunofluorescence study showing the signal of phosphorylated form of ERK1/2 (green) (**A**) and Akt (green) (**B**) in HaCaT cell treated with 10 μg/mL of CA extract for 15 min. Additionally, phosphorylation status of EGFR at tyrosine 1068 (**C**) and the expression of EGFR protein (red) (**D**) were examined. The nucleus of HaCaT cells were stained with DAPI (blue). Representative pictures were taken by a fluorescent microscope at 100× magnification (scale bar = 200 μm).

Additionally, when we detected the expression of Mcl-1, an anti-apoptotic protein in which its expression is regulated by the PI3K/Akt pathways, we found that CA extract induced the expression of this protein in a concentration-dependent manner (Figure 8A). Results from immunofluorescence study verified the findings from Western blot analysis and provided more information on the intracellular location of Mcl-1, which was likely to cluster in the mitochondria where this anti-apoptotic protein normally functions (Figure 8B).

3.5. Suppression of ERK1/2 and Akt Activation by Specific Inhibitors Attenuates CA Extract-Induced HaCaT Cell Monolayer Wound Healing

To further confirm the possible mechanism of action of CA extract, we designed additional experiments by using a MEK1 inhibitor (U0126) and a PI3K inhibitor (LY294002) to verify that ERK1/2 and Akt kinases are responsible molecular players in stimulating HaCaT cell proliferation and survival in response to CA extract. Data from Western blot analysis revealed that CA extract could strongly activate ERK1/2 and Akt, but not other MAPKs, including p38 and JNK (Figure 9A,B). As expected, U0126 could specifically inhibit CA extract-induced ERK1/2 phosphorylation (Figure 9C), whereas LY294002 could specifically block Akt phosphorylation (Figure 9D). Moreover, when U0126 and LY294002 were combined, the activation of ERK1/2 and Akt in HaCaT cells stimulated with CA extract was completely inhibited (Figure 9E).

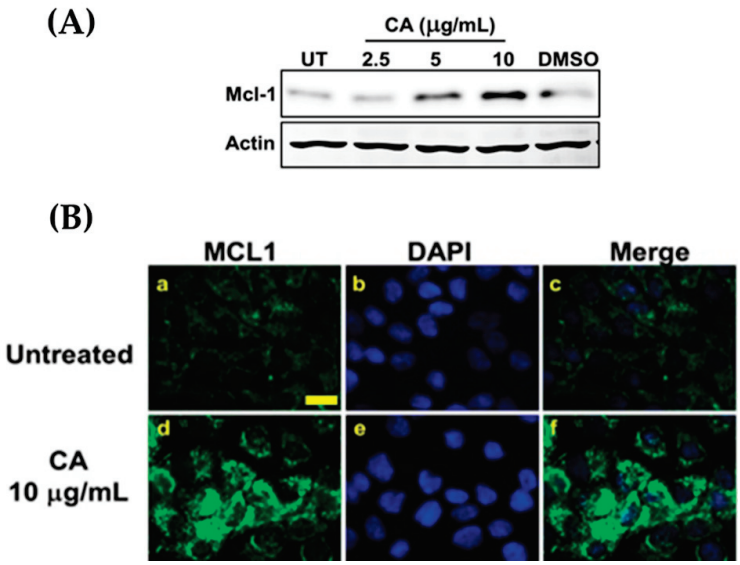

Figure 8. Effects of CA extract on Mcl-1 expression in HaCaT cells detected by Western blot analysis (**A**); and immunofluorescence study (100× magnification, scale bar = 200 μm) (**B**). Data were obtained from three individual experiments.

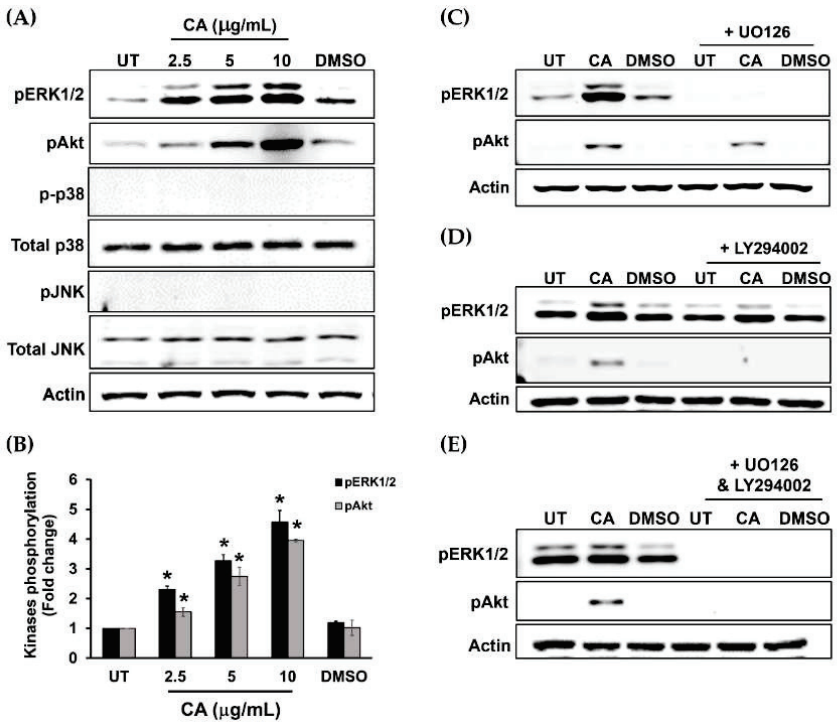

Figure 9. U0126 and LY294002 inhibited CA extract-induced ERK1/2 and Akt phosphorylation. (**A**) Western blot detecting phosphorylated ERK1/2 and Akt in CA-extract-treated HaCaT cells. (**B**) Quantitative analysis of the fold change of ERK1/2 and Akt kinase phosphorylation. (**C**) Western blot analysis for phosphorylated ERK1/2 and Akt in CA-extract-treated HaCaT cells with U0126. (**D**) Western blot detecting phosphorylated ERK1/2 and Akt in CA-extract-treated HaCaT cells with LY294002. (**E**) Western blot detecting phosphorylated ERK1/2 and Akt in CA-extract-treated HaCaT cells with U0126 plus LY294002. Data from three individual experiments present mean ± SD; * $p < 0.05$.

We next performed a functional test to evaluate whether suppression of growth and survival signaling by U0126 and LY294002 can attenuate healing-enhancing effects of CA extract. Scratch wound healing assay revealed that the effects of CA extract on stimulating HaCaT cell migration were remarkably blocked when U0126 alone, LY294002 alone, or the combination of these two inhibitors were present (Figure 10).

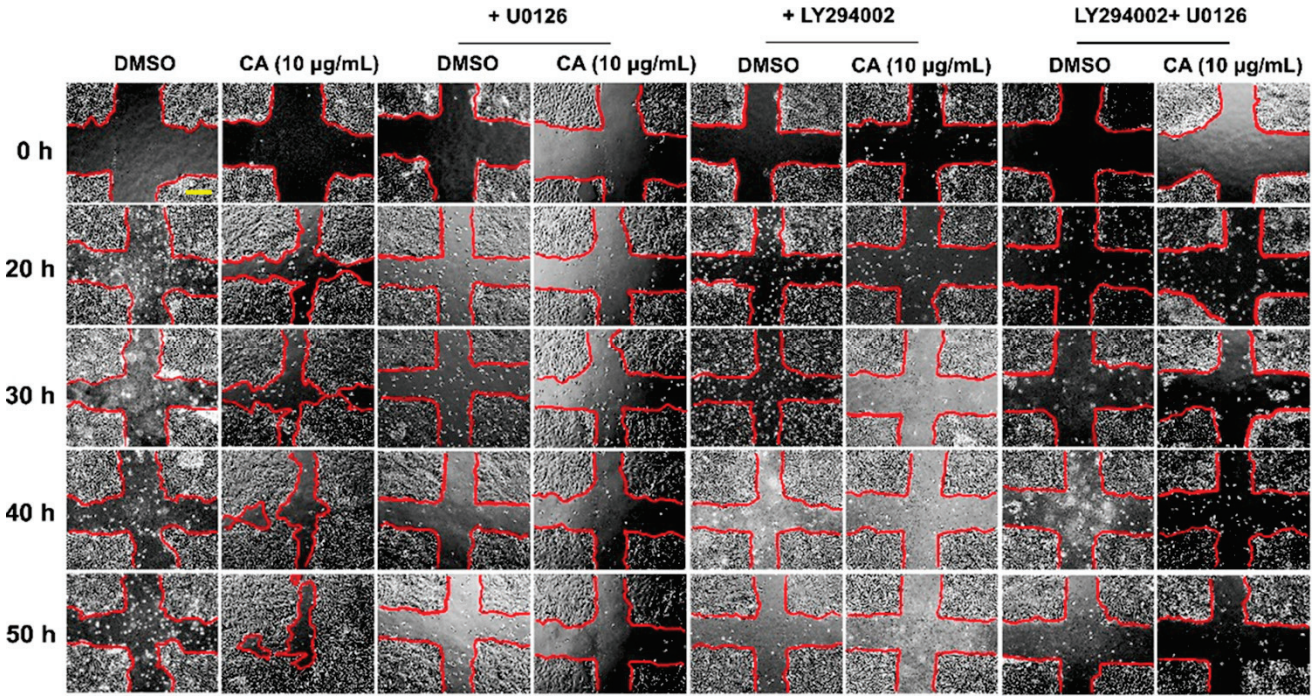

Figure 10. U0126 and LY294002S strongly inhibited the healing-promoting activities of CA extract. Phase-contrast microscopy at 10× magnification (scale bar = 200 μm) was performed to monitor the rate of migration into the wounded site of HaCaT cells treated with CA extract in media containing U0126, LY294002, or the combination of U0126 and LY294002 over the course of 50 h. Regions confined by the red lines indicate the space with no cell occupation. Data are from 3 different experiments.

4. Discussion

Here we studied *Curcuma amarissima* (CA) ethanolic extract by focusing on its wound healing activities by using HaCaT cell monolayer as a study model, since this cell type is derived from human skin. In particular, we attempted to investigate it to gain concrete evidence for its molecular mechanisms. We first performed a cell viability test by MTT assay to select a range of concentrations that were not toxic to a human epithelial keratinocytes (HaCaT) cell line. Interestingly, results from this experiment where we treated cells with CA extract in FBS-free media clearly showed that CA extract may be able to promote the viability of HaCaT cells. However, this positive effect of CA extract on cell viability was not observed when the treatment system was in complete media where it contained 10% fetal bovine serum (FBS). This phenomenon may be caused by high amount of growth factors in culture media that helps maintain a degree of cell viability so high that it conceals the effects of CA extract. Serum-free condition lacks growth factors, thus allowing its effects on cell viability enhancement to stand out. Data from MTT assay suggest the possibility that CA extract may be able to induce specific cellular events that increase the metabolic activity of the cell, or it may have resulted from increased cell proliferation. As expected, our results from colony-forming assay demonstrated that CA extract could dramatically stimulate the growth of HaCaT colony, suggesting that CA extract may contain potential active constituents that can regulate cell proliferation. Data from crystal violet staining and cell counting clearly confirmed that CA extract promotes cell proliferation to

increase a significant number of keratinocytes over time. The possible mechanisms of CA may be similar to those of several growth factors that are critical factors to stimulate the proliferation of human keratinocytes and eventually contribute to efficient healing [35,36]. We then performed a functional test by using a scratch wound model and discovered that CA extract induced a drastic increase in the rate of cell monolayer wound healing. According to our findings, it provides a close correlation between the closure of scratch wound and an increase in HaCaT cell number, suggesting that a contribution to wound healing of CA extract mainly derives from increased cell proliferation.

On the basis that the mitogen-activated protein kinase (MAPK) signaling normally plays crucial roles in cell migration and proliferation regulation [11,12,37,38], we therefore examined whether CA extract contributes to HaCaT cell proliferation through activation of this signal transduction pathway. Interestingly, we disclosed that CA extract stimulated ERK1/2 phosphorylation, and when the phosphorylation of this kinase was inhibited with U0126, the migration rate of HaCaT monolayer was dramatically suppressed. These data strongly suggest that CA extract may possess wound-repairing effects by enhancing cell proliferation and migration, at least in part through ERK1/2 activation. To support our statement about the involvement of ERK1/2 in keratinocyte, many studies previously demonstrated that ERK phosphorylation promoted the migration of this cell type [39,40]. Although ERK1/2 was activated by CA extract, the phosphorylation of p38 kinase and JNK kinase was not affected. These results suggest that not all MAPK signals are involved in CA extract-induced HaCaT cell monolayer healing and indicate that ERK1/2 is the primary signal pathway activated by CA extract. Besides ERK1/2, the phosphatidylinositol 3-kinase (PI3K/Akt) signaling transduction pathway is well characterized to be responsible for migration of many cell types [41–43]. Therefore, we determined the effect of CA extract on Akt phosphorylation status in human keratinocytes. Like ERK1/2, we observed a strong increase in phosphorylated Akt level in HaCaT cells incubated with CA extract. Consistent with results from Western blot, an increase in phosphorylation of Akt in individual cells was also confirmed by immunofluorescence study. Additionally, the involvement of this signaling pathway in HaCaT cells induced by CA extract was verified by wound closure assay where a PI3K inhibitor, LY294002, was present. When both U0126 and LY294002 were combined, no monolayer wound healing was evident, indicating that the wound healing activities of CA extract in human keratinocytes are regulated through the activation of the MAPK and PI3K/Akt signal transduction pathways. In addition to the effects on cell proliferation, CA extract has an influence on cell survival by increasing the production of Mcl-1, which is a key anti-apoptotic protein. Mcl-1 production is under the influence of the PI3K/Akt pathway [44]. Moreover, Mcl-1 protein can be upregulated by some cytokines such as IL-6 [45] and growth factors such as EGF, which conveys the cellular signal to control Mcl-1 translation via the MAPK pathway [46,47]. We observed that Mcl-1 was upregulated in individual cells treated with CA extract, and the localization pattern of Mcl-1 suggests that this protein resides in the mitochondria of the cells. Induction of strong expression of Mcl-1 anti-apoptotic protein suggests that CA extract helps increase cell survival of keratinocytes. However, transactivation of epidermal growth factor (EGFR) was not affected by CA extract, indicating that CA extract does not stimulate ERK1/2 and Akt phosphorylation via the EGFR signaling cascade. The activity of CA extract in inducing an increase in cell proliferation and cell survival may be caused by its curcuminoids, including curcumin and demethoxycurcumin. It is evident that curcumin (predominantly found in *Curcuma longa*) stimulates fibroblast proliferation, enhances the formation of granulation tissue, and promotes the contraction and epithelialization of wounds [48,49]. Moreover, the finding that CA extract contains ferulic acid strengthens the possibility to utilize this plant for wound healing since ferulic acid has been documented to be beneficial for skin repair, and this compound has recently been incorporated into the formulation of wound healing [50]. Therefore, our study suggests that CA extract possesses its wound-healing effects, at least in part, through the action of curcuminoids and ferulic acid.

However, our study, using HaCaT cells as a model, has some limitations that require future investigations by other models since HaCaT contains certain genetic alterations that may cause the cell to respond to certain stimuli in a different manner compared to human keratinocyte in actual physiologic conditions. For this reason, human primary keratinocyte should be used to verify the effects of CA extract and its active compound on proliferation and survival. Nevertheless, the use of normal primary human keratinocytes can be limited by the complexities involved in their recovery from donors, cultivation, and limited number of passages. Moreover, primary keratinocytes in vitro have little in common with ordinary naive keratinocytes in vivo under homeostatic conditions. Considering that wounding is a stress that requires several different complex series of communicating processes to respond to various stimuli and to heal the wound [51,52], it needs specific models that can be able to address the promoting effects of a certain agent on wound healing. To overcome these limitations, animal models and clinical trials in humans should be performed in the future to achieve our complete understanding of physiologic and pathologic processes as well as translational efficiency. Altogether, our study revealed that CA extract can potentially trigger and strengthen the molecular healing cascades in human keratinocytes. These events are of great interest for the development of CA as an alternative option for wound healing occurring in some diseases, such as complicated diabetes mellitus, which is less sensitive to treatment by growth factors [53].

5. Conclusions

Our present study provides evidence that *Curcuma amarissima* (CA) possesses pharmacological properties in activating human keratinocyte proliferation and survival through its ability to strongly stimulate the phosphorylation of ERK1/2 and Akt kinases. These properties are generally required for promoting wound healing. We demonstrate that this plant contains curcumin, demethoxycurcumin, and ferulic acid, which are potential active compounds reported to be able to promote skin regeneration. Our discovery provides information beneficial for potential uses of CA in regenerative medicine. When mechanisms of action of CA in wound healing is completely defined, and further investigation in animal and human models are done, this plant may be an excellent candidate for the development of a wound-healing agent.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10040289/s1, Figure S1: Chromatographic fingerprint analysis of the ethanolic extract from *Curcuma amarissima* (CA) by high-performance liquid chromatography (HPLC), Figure S2: The HPLC profile of standard ferulic acid and the extract from *Curcuma amarissima*.

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Review

A Descriptive Overview of the Medical Uses Given to *Mentha* Aromatic Herbs throughout History

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Simple Summary: Mints are aromatic herbs with a millenary tradition of use for diverse medicinal purposes since ancient civilizations, and they are still presently used in different clinical practices. Mints have been used since ancient Babylon, but it was in Classical Antiquity that their medical uses flourished, with major contributions from Pliny the Elder. In the Middle Ages, the increased knowledge surrounding mints came from Byzantine physicians, while, in the Modern Age, technological developments allowed the production of mint-based products, such as extracts and essential oils, which have become part of elaborate galenic formulas employed by an increasing number of physicians, and have also stimulated both scientific and artistic interests alike. In present-day medicine, several mints and mint-based products are being researched as potential therapeutic alternatives for many diseases, while also being vastly employed in food and cosmetic industries.

Abstract: Mints have been among the most widely used herbs for medicinal purposes since ancient civilizations. They are still presently used for numerous purposes, including non-medicinal, which makes them economically relevant herbs. Information regarding the medical and scientific uses given to mints throughout history are vastly scattered and/or incomplete. The aim of this paper is to provide an extensive descriptive overview of the medical uses given to these herbs, highlighting both the authors in medical culture responsible for their dissemination, as well as their major galenic formulations. Databases on medical science, reference textbooks on medical history, botanics (aromatic herbs), and pharmacognosy were consulted. The use of mints remotes to Classical Antiquity, with major contributions from Pliny the Elder. In the Middle Ages, the increased knowledge surrounding mints came from Byzantine physicians, while, in the Modern Age, technological developments allowed the production of mint-based products which have become part of elaborate galenic formulas employed by an increasing number of physicians, as well as have also stimulated both scientific and artistic interests alike. In present-day medicine, several mints and mint-based products are being researched as potential therapeutic alternatives for many diseases, while also being vastly employed in food and cosmetic industries.

Keywords: *Mentha* L. genus; aromatic herb; history; pharmacognosy; therapeutics

1. Introduction

Medicinal plants have been at the forefront of most medical therapies for many centuries and have been used in many societies worldwide. A large number of medical treatises have established several plant-based medicinal products as the most important components of the available therapeutic arsenal. Members of aromatic mint herbs have been among the most widely used plants throughout history for a multitude of medicinal purposes. This paper provides a thorough review of the medical uses given to mint herbs in western medicine from ancient civilizations to modern day medicine and

provides recent scientific data to support the reasons behind the longevity of their use. Mint herbs consist of perennial aromatic members of the Lamiaceae family, the *Mentha* L. genus, classified into 42 species, 15 hybrids, and hundreds of subspecies, varieties and cultivars. Among the best-known mint species, we find watermint (*Mentha aquatic* L.), spearmint (*Mentha spicata* L.), wildmint (*Mentha arvensis* L.), horsemint (*Mentha longifolia* (L.) L.), pennyroyal (*Mentha pulegium* L.), and peppermint (*Mentha x piperita* L.), the last of which is a natural sterile hybrid of watermint and spearmint [1,2]. While most mints have been known to man since ancient times, it was not until 1696 that peppermint was described by English Botanist John Ray (1627–1705) in his *Synopsis Methodica Stirpium Britannicarum* [3].

Most mints invariably grow in moist environments near ponds, lakes, and rivers and require partial shade, although some species grow well in warm environments [4]. Interestingly, one of the first written references to mints, the poem *Theriaca* by Nicander of Colophon (197 BC–170 BC), Greek poet and physician, alludes to their habitat and describes them as 'delighting in gleaming rivers' [5]. Sterile mint hybrids, such as peppermint, only display vegetative reproduction, while the majority display sexual reproduction and contain both male and female organs [4]. It is likely that mints' sexual dimorphism justified the comparison made between pennyroyal and the fictitious magical plant *Pantagruelion*, as featured in François Rabelais' *Gargantua and Pantagruel* [6]. The first attempt to classify them from a botanical perspective came from Pedanius Dioscorides (c. 40–90), the renowned Greek physician and herbalist, whose teachings became a reference from classical antiquity well into the Renaissance period. A more formal classification was proposed several centuries later by Carl Nilsson Linnæus (1707–1778) in his *Species Plantarum* (The Species of Plants) published in 1753 [7], which has been further perfected in recent years based on the genetic relations between these species [8]. To contribute to the complexity of the mint taxonomy, all the different species are referred to in many medical treatises as simply mint, which prevents a more precise appreciation of the uses given to each particular species. From a pharmacognosy perspective, the therapeutic value of herbs lies in aerial parts, which can be dried and ground into powder or used fresh. If used fresh, aerial parts can be subjected to water or steam distillation to produce an essential oil that is extracted [4].

The name of the mints' genus, *Mentha*, was probably coined by the Greek philosopher Theophrastus (371 BC–287 BC), who described several species from botanical and agricultural standpoints in his *Enquiry into Plants* [9]. The name *Mentha* probably alludes to Minthe, a figure of Greek mythology, although different ancient texts describe different versions of her myth. According to the myth told in the didactic epic *Halieutika* (On Fishing) by Oppianus of Corycus (fl. 2nd century), the Greek-Roman poet contemporary of emperor Marcus Aurelius and of Galen, Minthe was a nymph of the river Cocythus and wife of Aidoneus. But when Aidoneus raped Persephone, of the Aetnaean hill, Minthe, overtaken by jealousy, claimed to be nobler of form and more excellent in beauty than Persephone, and, for this, she was trampled upon and destroyed by Demeter, Persephone's mother, and from the earth sprang the herb that carries her name [10]. In the versions told by Strabo (c. 64 BC–24), the Roman geographer, and by the Roman poet Ovid (43 BC–17), Minthe was beloved by Hades, and it was Persephone, the god's jealous wife that destroyed the nymph [11,12].

The oldest written records of mint herbs are attributed to King Hammurabi of ancient Babylon (1800 BC), who prescribed them for medicinal purposes, namely gastrointestinal [13]. Mints are also referred to in the Ebers papyrus and on the walls of the temple of Horus in Edfu [14,15]. A great deal of knowledge on the mints' usage was acquired during classical antiquity by Greek and Roman philosophers, especially Gaius Plinius Secundus (Pliny the Elder, 23–79), who described most of the medical uses that would be given to these herbs throughout the history of western medical practice in his *Naturalis Historia* (Natural History) [16], an important work that would be the reference for several posterior medical texts. Knowledge of these herbs spread throughout the Middle East, partly due to the conquests of Alexander the Great (356 BC–333 BC) and later to the Crusades [17]. Given their many uses, mints became valuable herbs and were probably used as currency by the Pharisees, as can be inferred from two passages from the Bible: '(. . .) Woe to you, scribes and Pharisees, hypocrites! For you tithe mint, dill, and cumin, and have left undone the weightier matters of the Law: justice,

mercy, and faith (. . .)' (Matthew 23:23) and '(. . .) But woe to you Pharisees! For you tithe mint and rue and every herb, but you bypass justice and the love of God (. . .)' (Luke 11:42) [18]. Several references are also found in the Babylonian Talmud, a compilation of Jewish teachings on several knowledge areas that contains many pieces of practical advice of a medical nature [19]. In the Middle Ages, medical and scientific learning shifted to Constantinople, the capital of the Byzantine Empire, with the majority of important references to mints coming from Islamic physicians, such as Ibn Sinna (Avicenna, 980–1037), author of *Canon of Medicine* [20]. In Christian Europe, medical knowledge was kept mostly in universities and monasteries by physician-monks, where the teachings of classical philosophers were still followed [17]. Charlemagne (c. 747–814), leader of the Holy Roman Empire and founder of the first medical school in Salerno in the 9th century, ordered medicinal plants, in which mints were already included, to be cultivated inside state-owned land, as stated in his *Capitulare de villis* [21]. The Renaissance period allowed a change to medico-scientific paradigms and the progressive abandonment of empiric frameworks as regards medical plants to embrace more experimentally ascertained facts. This was also a period of artistic flourishment, when the printing press was invented which facilitated the dissemination of literary works [22]. Incidentally, one of the first *incunabula* (i.e., earliest printed books, between 1450 and 1500), the famous and obscure *Hypnerotomachia Poliphili* (Poliphilo's Strife of Love in a Dream) attributed to the Italian Dominican priest Francesco Colonna (c. 1433–1527), features several mint species among the numerous botanical varieties described in the story's oneiric landscapes [23]. The 16th century was marked by the rupture with the orthodox medical doctrine based on the theory of humors, and a new doctrine emerged called *nova medicina*, which was rooted in alchemy, astrology, magic, and natural philosophy. The advent of iatrochemistry made possible for new plant extracts and essential oils to be prepared, some from the recently discovered New World, which increased the number and variety of available medicine products [24]. The development of pharmaceutical technology from the 19th century onward has largely contributed to the diversification of mint-based products [25]. Peppermint essential oil, for example, became such a popular medicine in the 19th century that it found its way into the post-impressionistic painting *Still Life with Peppermint Bottle* by French painter Paul Cézanne [26]. Apart from being a source of inspiration to artistic expression, mints also aroused curiosity in the emerging scientific fields. For example, one mint species was used by Joseph Priestley (1733–1804) in his pioneering studies on photosynthesis [27], while Charles Darwin (1809–1882) used peppermint essential oil for his studies on botanics [28]. Mint active compounds concentrate mainly in the leaves of herbs, which can be used by dry grounding them into powder or fresh and being subjected to solvent extraction or steam distillation, the latter of which produces mint essential oils. These oils contain a variety of volatile compounds and are widely used in food, cosmetic, and perfume industries, largely for their flavoring, fragrance, and preservative properties, which make them very economically valuable. The cultivation and processing of mint herbs is a very large business and one that is responsible for a considerable part of the economy of many countries [4].

Most of the data regarding the medical and scientific uses given to mints throughout history are vastly scattered and/or incomplete. The aim of this paper was to provide an extensive descriptive overview of the medical uses given to these herbs, highlighting both the authors in medical culture responsible for their dissemination, as well as their major galenic formulations. The next section references these authors and respective written sources in chronological order (Table 1), together with a concise and up-to-date appreciation of these medical uses in light of ongoing scientific research (Table 2). A comprehensive review of the composition of the mint herbs and a correlation with their ancient/actual medical properties is beyond the scope of this paper. Databases on medical science (Pubmed, Springer Link, Google Scholar, Internet Archive, U.S. National Library of Medicine) were searched using combinations of the following keywords: "mentha", "mint", and its variations to include the several species' names, "medical" and "history". Reference textbooks on medical history, botanics (aromatic herbs), and pharmacognosy were also consulted. The literary works of the most relevant authors were accessed and analyzed as regarding the previous keywords. From the numerous

medical/science research papers accessed, the most relevant ones for the historical discussion comprised in this paper were selected. Given that some authors of the many analyzed written sources do not clearly identify the mint species used, the term "mint" will be used throughout this paper with the meaning "unknown species". Nevertheless, whenever possible, the names of the concerned species will be provided.

2. Medical Applications

2.1. For Scenting and Perfuming

Probably the most recognizable quality of mints is their appealing fragrant scent, which is why they have been used since early times in embalming funerary rites. The embalming practice continued until the Middle Ages in Christian Europe but was reserved to the elite. In fact, mint was recently found among the embalmed remains of King Richard the Lionheart (1157–1199) [29] and of John Plantagenet of Lancaster, first Duke of Bedford (1389–1435) [30]. The purpose of using aromatic herbs, such as mints, in these practices was not only to create conditions for long-term body conservation but to also confer it a good odor, similarly to the body of Christ (i.e., the sanctity odor) [29].

Mints were also employed to manufacture perfumes and to mask distasteful substances in medicines. For hygienic purposes, Paulus Aegineta (625–690), a Byzantine Greek physician and compiler of Greek–Roman medicine, best known for his encyclopedia *Medical Compendium*, mentioned the use of ground pennyroyal in a mint decoction, among several ingredients, to mask the displeasing taste of medicinal draughts [31]. Peppermint essential oil is featured in several formulations containing either laudanum (i.e., an opium tincture) or morphine (i.e., the main component of opium). Albert Ethelbert Ebert (1840–1906), a prominent pharmacist of the late 19th century, featured peppermint essence in *The Standard Formulary* as an ingredient of a complex formulation in which morphine was included [32]. Similar uses were later carried out to mask the odor of fish oil [33], cod liver oil [34,35], and laudanum. An example of the last case was the inclusion in the infamous nostrum *Shiloh's consumption cure* to be used for colds, coughs, bronchitis, asthma, and irritation of the throat [36]. Unquestionably, the most explored mint compound for scenting and perfuming is menthol. This is easily explained by its capacity to induce the perception of coolness, which can strongly affect cognition, emotion and behavior [37]. It is legitimate to interpret that, given the inflammatory component of many diseases, manifested topically with redness and warmth, a "cooling" or "fresh" sensation would be logically sought for to calm the effect. Furthermore, the known local menthol anesthetic seems to have been also an important reason to include these herbs in medical recipes.

2.2. For Gastrointestinal Disorders

The medical use of mints for gastrointestinal affections is present in the works of most philosophers and physicians who came across them, from classical antiquity to present-day medicine. Mints have been consistently referred to as possessing anti-emetic and carminative properties, and being used to facilitate digestion and assist in the treatment of gastrointestinal disorders. Pliny was among the first to document these properties and, quoting Democritus, wrote that mints were a suitable treatment for vomiting [17]. Aëtios of Amida (502–575), a Byzantine physician and writer, credits Kyrillos, the archbishop, with a recipe of a digestive composed of a mixture of plants, including pennyroyal macerated in vinegar [38]. Paulus Aegineta left many references to the digestive benefits of mints. For stomach problems, he advised drinking 'a draught of juice of endive sprinkled with mint' or 'a mixture of juice of kernel, pomegranate and mint' [31]. Centuries later, Hildegard von Bingen (1098–1179), a German Benedictine abbess and polymath, left many texts about the use of medical plants in her *Physica*. To adding to her theoretical knowledge, she gained practical experience in the use of the plants that she grew in her monastery's garden. To ease digestion, she advised consuming watermint and spearmint for they 'warm the stomach' [39]. Trota, a prominent figure of the medical school of Salerno in the 12th century, was another female healer and medical writer to have contributed

to extend knowledge on female healthcare. In her *Trotula*, a famous compendium of texts on women's medicine, she detailed several recipes involving mints [40]. For constipation one recipe consisted on cooking mint in honey and water, a beverage that was to be drunk by after bloodletting. Another recipe consisted in a mixture of wild celery, mints, cowbane, mastic, cloves, watercress, madder root, sugar, castoreum, zedoary, and gladden. This mixture was to be made into a very fine powder and be given with wine to relieve abdominal distension caused by trapped gas during pregnancy and the consequent risk of miscarriage. Another mention in the *Trotula, Potio Sancti Pauli* (Saint Paul's Potion), a potion including horsemint, was made to diverse gastric ailments. With the extended use of mints throughout Europe, several recipes began to appear among the royal apothecaries' arsenals. For example, a recipe for a plaster made of wheat bread, cumin, wormwood, mint, and rose leaves figures in the long list of medicines to Katherine Neville, Duchess of Norfolk by John Clerk (15th century), king's apothecary to Edward IV, which represents both the fear of epidemics and the need to correct the excesses of the aristocratic lifestyle [41]. Mint medicines also appear in the apothecary's list for Anne of Bohemia, first wife of King Richard II. Although the use given to mints in these lists is not specified, the intended treatment of gastrointestinal disorders is probable [42].

Theophrastus von Hohenheim (Paracelsus, 1493–1541), the German-Swiss physician and alchemist, is credited with bridging medical practice and chemistry fundaments. For difficult digestions, Paracelsus suggested consuming a mixture of mint water and syrup of gillyglower [43]. Garcia de Orta (1501–1568), a Portuguese physician and herbalist who pioneered tropical medicine while working in the empire's eastern colonies, wrote an important medical treatise on pharmacognosy entitled *Colóquios dos simples e drogas da India* (Conversations on the simples, drugs and medicinal substances of India). In this work, Garcia de Orta advised mixing mint water and mastic powder for 'vomiting and weakness of the stomach' [44], which would be later referenced by the also celebrated Portuguese physician and naturalist Cristóvão da Costa (1515–1594) in his *Tractado de las drogas y medicinas de la Indias Orientales* (Treatise of the drugs and medicines of the East Indies), based on Garcia de Orta's own work [45]. Prosper Alpinus (1553–1617), a Venetian physician and botanist, referenced a treatment for bile vomiting consisting in the administration of 'sub-acid fruits, juice of wormwood or of mint in wine' [31]. Herman Boerhaave (1668–1738), the prominent Dutch physician, was also interested in mints for their usefulness in digestive ailments. In his *Materia medica* (On Medical Material), he wrote about watermint and peppermint as anti-emetics [46]. Thomas Sydenham (1624–1689), the prominent English physician, used mint water as a solvent for several anti-emetic agents [47].

The carminative (i.e., reliever of flatulence) and spasmolytic properties of mints were also much appreciated. Paulus Aegineta provided a recipe for constipation in children, for which he advised rubbing the abdomen with a mixture of mint and honey [31]. Robert Burton (Democritus Junior, 1577–1640), Oxford scholar and author of the celebrated *The anatomy of melancholy*, described watermint as an effective carminative [48]. The prominent English physician and founding member of the Royal Society, Thomas Willis (1621–1675), wrote recipes using mint as an anti-emetic and antispasmodic in his *Dr. Willis's Receipts for the Cure of All Distempers* [49], while Sydenham used watermint to relieve the so-called 'iliac passion' (i.e., intestinal volvulus) [47]. Reverend Joseph Townsend (1739–1816), English physician and vicar, listed peppermint as an effective spasmolytic in his *Elements of Therapeutics: or, a Guide to Health; being Cautions and Directions in the Treatment of Diseases* [50]. In the late 19th century, a nursing book by Isabel Robb (1860–1910), a nurse theorist, mentions the internal application of peppermint water to ameliorate colic in infants [51].

The gastrointestinal usefulness attributed to mints has been uncovered in recent scientific publications. Mint oils possess substances that increase gastric emptying to improve digestion [52] and relax the bowel [53,54]. Its antiemetic properties are of considerable magnitude as they can reduce postoperative, chemotherapy-induced nausea and vomiting [55,56]. Their spasmolytic properties are known to relieve symptoms of irritable bowel syndrome [57–59]. They are safe to be used in endoscopic procedures, as a suitable alternative to conventional spasmolytics, and increase the diagnostic sensitivity of the procedure itself [60].

References to intestinal infections, especially of parasitic etiology, can be found in numerous medical treatises, and mints appear as useful therapeutic herbs. Pliny advised taking dry powdered mint in water to expel intestinal worms [17], a reference which was imported into one of the most read works in the Middle Ages, *Macer Floridus*, a hexametric poem on medicine and botanics, presumably authored by Odo de Magdunensis (1070?–1112?) [61,62]. Dioscorides recognized the ability of spearmint to kill roundworms [63]. The Babylonian Talmud advises eating pennyroyal with seven white dates to kill intestinal worms caused by eating raw meat [64]. Paulus Aegineta advised the external application of 'mint or gith in rose-oil' to the navel for worm infection and to also give 'sebesten plums and mint' to aid low fevers brought up by worm infections [31]. Alexander of Tralles (525–605), a prominent physician from the early Byzantine period and an apparently accomplished helminthologist, also listed watermint among several effective antihelminths [65]. Centuries later, the *Regimen Sanitatis Salernitanum* (The Salernitan Rule of Health), a medieval didactic poem from the Salerno medical school, briefly refers to mints as antihelmintic herbs [66].

The use of mints to treat cholera is described in several medical texts, with the first being once again attributed to Pliny [17]. For cholera, Paulus Aegineta advised drinking 'juice of pomegranate sprinkled with mint' [31]. Sydenham used mint as a component of a nourishing drink for cholera patients [47]. Physician William Currie (1754–1828) who, in the 19th century described the disease in his work *Of the Cholera*, also used mints as therapeutic herbs. He also recommended using peppermint water as a palliative treatment for digestive and spasmodic problems and mentioned the activity of mints' oils against a multitude of pathogenic microorganisms, including *Vibrio cholera*, the causative agent of cholera [67]. Recent studies have shown that the essential oils of several mint species have shown in vitro activity against *Echinococcus*, *Trichostrongylidae*, and roundworm parasites [68–70], as well as in vivo activity against *Giardia* and *Entamoeba* species, to name just a few [71].

2.3. For Reproductive Purposes

Mints have had vast reproduction applications for not only female hygiene and contraceptive purposes but also for their abortifacient properties. The first records on female health date back to ancient Greece, where contraception was almost limited to the so-called "barrier methods". Mints were added to pessaries, especially balls of wool, inserted in the female reproductive tract, probably for the cooling/calming sensation they evoked [31,72]. As for hygienic measures, mints were used for washing female genitalia after coition. Soranus of Ephesus (98–138), a Greek physician best known for his gynecological and obstetric work *Gynecology*, prescribed pennyroyal for sitz baths [73]. Similarly, John of Gaddesden (c. 1280–1361), a famous practitioner in 14th century England and the author of *Rosa Medicinae*, advised using watermint for the hygienic washing of female genitalia [74].

The best-known use of mint herbs for female health undoubtedly comes from their recognized effect of increasing uterine contractile strength (i.e., oxytocic). They were used as emmenagogues, especially in the clinical context of dysmenorrhea. Galen (129–c. 210), in his *On the Mixtures and Powers of Simple Drugs*, suggested using watermint and pennyroyal as complementary treatments to bloodletting, a popular treatment for plethoric ailments, and mentioned that they 'bring on an abundant menstrual flow' [75]. Centuries later, Luis de Mercado (1525–1611), physician to Phillip II of Spain, also listed pennyroyal as an herb to relieve menstrual retention [76]. The most popular mint for its abortifacient properties was pennyroyal, which has been featured in numeral medical treatises, as well in mythological texts. In the Homeric *Hymn to Demeter*, there is a reference to a recipe for *kykeon*, a porridge containing honey-sweet wine, barley, water, and pennyroyal that was drunk by the goddess Demeter herself. This reference is probably a connotation between pennyroyal and female health and sexuality, but whether it precedes the medical use of the herb or was responsible for it is still a matter of discussion [77]. Pliny described pennyroyal and other mints as being capable of increasing uterine contractions and to 'help expel the placenta and a dead fetus' [17], a belief later repeated by Dioscorides [78]. Quintus Serenus Sammonicus (d. 212), the Roman author of *Liber Medicinalis*, a didactic poem on medicine, advised administering pennyroyal in tepid water to induce

abortion in women with pregnancies less than 1 month old and whose 'embryo was weak' [79], a practice also mentioned in Odo de Magnudensis' work [61]. Pennyroyal is referenced in *Acharnians*, *Peace*, and *Lysistrata*, three plays by the celebrated dramaturge Aristophanes (c. 450 BC–c. 388 BC). In *Peace*, pennyroyal is suggested to have contraceptive properties [79], and in *Acharnians* and *Lysistrata*, the blossoming of pennyroyal is mentioned as a metaphor for pubic hair [80–82]. Trota advised the anointment of pennyroyal oil on a cloth to be placed in the vagina to induce receding of prolapsed uterus caused by traumatic coitus due to the 'excessive size of the male member'. Pennyroyal also appears in Trota's work as an herb to be applied in a bath, among several others, which 'flegmatic and emaciated' women who could not conceive should take to increase the chances of fertilization. Trota also described several recipes for stimulating menses. In a simple recipe, honey was to be cooked in mint water; mint also appeared in the herb mixture *Tyriaca diathessaron*. A mixture of mint, pennyroyal, rue, red cabbage, leek, and salt would be cooked together in a plain pot, and be drunk in the bath. In addition, she advised the fumigation of cumin, fennel, dill, calamint, mint, and nettle, either individually or mixed. Furthermore, a mixture of ground castoreum, white pepper, costmary, mint, and wild celery in white or sweet wine to be drunk in the evening also appeared. To help women having difficulties to give birth, mint and wormwood powder would also be given, besides mint and other odoriferous herbs being applied to the cervix during the prepartum period. The beverage *Tyriaca magna Galeni*, taken with mint water, was also given to stimulate menses or parturition [40,83]. Centuries later, Hildegard von Bingen wrote in her *Physica* that eating pennyroyal 'acts to expel the afterbirth' that remained inside the uterus after delivering the fetus [39]. In the 17th century, James Primerose (d. 1659), English physician and notable opponent of William Harvey's Theory of Circulation, who dedicated a large portion of his career to gynecology and obstetrics, had also mentioned pennyroyal to be an abortifacient [76]. Nicholas Culpepper (1616–1654), physician, astrologer, and herbalist warned in his *A Directory for Midwives* 'give not [pennyroyal] to any that is with Child, lest you turn Murderess' [84]. The almanacs of two female authors of the 17th century England, Sarah Jinner of London (fl. 1658–1664) and Mary Holden of Sudbury (c. 1648–1726), transmitted classically-based medical cures for women, challenging the existing medical hierarchy [85]. Sarah Jinner, a student of astrology and a contemporary of Primerose, wrote the first series of almanacs that focused on female health and destined to transmit updated gynecological knowledge from the medical elite to common literate persons and rural physicians [86]. These almanacs contained herbal useful remedies for managing and treating gynecological disorders, among which a list of 'pills to expel a dead child' is included, several containing pennyroyal as an ingredient, which she also noted as being able to regulate menses [87]. Thus, pennyroyal became a household herb for inducing abortion and is reported to have been taken with gin during menstruation as recently as the 1950s [88]. The strong effect of pennyroyal is attributed to the oxytocic terpene pulegone, which has, nonetheless, an important hepatotoxic profile [89].

Regarding male health and fertility, several considerations regarding the possible effect of mints on sperm generation existed. Pliny wrote that adding watermint to milk prevented it from curdling and thickening or turning sour. Assuming that a similar effect would occur with semen, Pliny wrote that ingesting watermint could change the consistency of semen and, therefore, affect fertility [17]. Dioscorides also believed that mints, if taken in large quantities, changed sperm quality and affected erection [31]. Aëtius of Antioch (d. 367), Syrian bishop and physician, wrote that watermint consumption 'generates much semen, but of a feeble nature' [31]. Avicenna wrote about watermint as a spermicide, used as a female suppository before coition [74]. In contrast, Trota wrote that men should apply pennyroyal (presumably externally), among others, to increase fertility [40]. Thus, pennyroyal was a valued herb through the ages for female health, but, in the 20th century, its use has steadily declined mainly due to the creation of abortifacient drugs and to the increase in the awareness against this herb's toxicity profile, with only scarce records of its use being found today.

2.4. For Modulating Libido

Reports of mints' effects on libido are diverse and often controversial. This may be partly explained by the differences in used species and also in the quality and quantity consumed. An obvious consensus was reached by classical philosophers that watermint was aphrodisiac. This notion was shared by Aristotle, Dioscorides, Galen, and, centuries later, by the Persian polymath Muhammad ibn Zakariya al-Razi (Rhases, 854–925) [31] and Nicholas Culpeper in his *The English Physician Enlarged* [90]. Contrary records exist but, strangely enough, appeared only in the Middle Ages. Avicenna recommended taking watermint as a treatment to reduce sexual desire [91], and a similar description is present in Hildegard von Bingen's *Physica* [39]. Similarly, Trota wrote that mints could be given to placate the repressed sexual desire [40]. Later, the Italian physician Paolo Zacchia (1584–1659), considered one of the fathers of forensic medicine, believed, in accordance with Hippocrates' beliefs, that eating watermint was responsible for erectile dysfunction [92]. However, the mints' effect on libido have generated only a modest interest thereafter. Recent animal and clinical studies agree that ingestion of mints have an anti-androgenic effect in males, which might affect erection and may also decrease libido [93].

2.5. For Repelling Insects and for Animal Bites

Providing protection against the different elements of the natural world, including animals, was always a concern for human beings, and mints have been employed as insect repellants and adjuncts in animal bite treatments since ancient times. By quoting Xenocrates (396 BC–314 BC), Pliny advised smelling pennyroyal and placing it near patients with tertian fevers (probably referring to malaria) that were prevalent in Hellenistic Greece and Egypt [17]. Trota also mentioned the utility of medicinal drinks *Tyriaca diatesereon* and Saint Paul's potion for patients with quartan fever (once again, probably malaria) [40]. These practices would likely have been intended to prevent *Plasmodium* protozoa from spreading, the causative agents of malaria from mosquito bites based on herbs' insect repellant activity. Paulus Aegineta noted that spreading herbs, including pennyroyal, was useful for repelling reptiles [31]. It has already been established that *Mentha* herbs, especially pennyroyal, have repellant, larvicidal and growth/reproduction regulatory activities against a wide variety of insects, including the mosquitoes responsible for spreading malaria (*Anopheles sp.*), yellow fever, dengue (*Aedes aegypti*), and zika (*Culex quinquefasciatus*) [94–98].

Mints were also valued for their scent in the Black Plague pandemic of the Middle Ages, which decimated at least one third of the European population. During this period, a vinegar-based formulation against the Black Plague called the "Four Thieves Vinegar" is thought to have originated in Medieval France, created by thieves that plundered the dead and dying plague victims. It is thought that, because they smeared this formulation on their skin, they were able to come in contact with their victims without being themselves affected [99]. Mints do not appear in the original formulation but were added to later variations of this formulation [100,101]. In this time period, it was still believed that (infectious) diseases were spread by miasmas, disease carrying vapors that emanated from corpses and decaying matter or from the breath of infected persons, a theory introduced by Hippocrates and Galen [102]. The discovery that plague was transmitted by fleas carrying the causative microbe *Yersinia pestis* was made centuries later. For these reasons, it is more logical that mints were added for their ability to offset pestilent odors rather than for their flea-repellant property; in fact, recent studies suggest that mint oils show only a weak flea repellant activity [103].

For the purpose of dealing with animal bites, several records are noteworthy. Nicander of Colophon used mints to create a 'repellant stench' to chase off snakes [5], while Pliny wrote of watermint and pennyroyal's usefulness for snake, scolopendra, and scorpion bites [17]. Trota also suggests people drinking *Tyriaca diatesereon* who had been bitten by poisonous animals and rabid dogs, as well as applying it to the wound itself. She also advised using *Tyriaca magna Galeni* with added mint water to help with poisoned wounds [40]. It is noteworthy that later references to mints are lacking, and recent scientific publications that have addressed the usefulness of mint-derived products to counteract animal venoms are scarce and inconclusive [104].

2.6. For Respiratory Disorders

Mints were also used to control respiratory ailments, although literary sources are not abundant. Plutarch (46–119) in his *Moralia* wrote of the habit of Heraclitus, the Greek philosopher of Ephesus, of drinking cold water with spigs of pennyroyal before giving a speech [105]. Pliny made a similar reference and advised taking watermint juice 'when a person is about to engage in a contest of eloquence, but only when taken just before' [17]. For lower respiratory tract problems, Theodorus Priscianus (b. 300), a physician from the 5th century Byzantine empire, used pennyroyal in an herbal mixture for chestpain [38]. Aëtios of Amida wrote of a cough medicine containing pennyroyal in a mixture of pepper, hyssop, terebinth, fresh butter, and honey [38]. Gil Rodrigues de Valadares (Giles of Santarém, 1185–1265), a Portuguese Dominican friar and physician, wrote mint-based medicinal preparations, which can be found in *Códice Eborense CXXI/2-19*. For aphonia, Giles of Santarém prescribed a mixture of mint juice, ground pepper, malva seeds, egg yolk powder, and honey to be applied to the tongue [106]. Another interesting reference found centuries later is made to Jean-Paul Marat (1743–1793), the famous French physician, scientist, and politician from the French Revolution. Marat prescribed a mixture of mint and tolu balms and vegetable extracts, as well as a secret nostrum, to treat the Marquise de Laubespine from tuberculosis, a case that increased his popularity among French elites [107]. Research has shown that mints' compounds create the perception of nasal decongestion but also display bronchodilator and antitussive properties [108–110]. Indeed, the menthol-based Vicks® chestrub is extremely popular in the United States for treating common colds [111].

2.7. For Cardiovascular and Urinary Disorders

There are only a few references made to using mint herbs for cardiovascular and urinary systems. Pliny wrote of pennyroyal as a diuretic and as one capable of removing bladder stones [17], an idea also defended by Aulus Cornelius Celsus (Celsus, c. 25 BC–c. 50) in regard to spearmint [112]. Later, Trota also advised using horsemint and pennyroyal in baths or fumigations to help with strangury (i.e., slow and painful discharge of small volumes of urine), likely due to their diuretic effect [38]. Interestingly, recent studies have shown that menthol does indeed have beneficial effects on the urinary system.

For example, menthol administration is associated with an improved inflammatory profile and clinical manifestations in female patients with interstitial cystitis [113]. As for their cardiac effect, Avicenna wrote of watermint in his opus *Canon of Medicine* as a valuable herb for heart conditions, especially palpitations [114]. Recent research suggests that the *Mentha x villosa* species has a hypotensive effect through bradycardia and vasodilation [115]. Although several mint substances may display this vasoactive effect, it has definitely been detected in menthol [116].

2.8. For Pain and Inflammation

Although the pathophysiological mechanisms of inflammation were not uncovered until the 20th century, its central concept was understood many centuries before by Celsus [117]. Accordingly, mints were used either topically or systemically to control the inflammatory manifestations of several diseases, including pain, erythema, and fever, since classical antiquity. Pliny used mints in formulas for cases of ophthalmic and oral inflammation [17]. The Talmud mentions using pennyroyal in a mixture of boiled herbs to be applied to the scalp to 'soften the skull' prior to cranial surgery, which would suggest that pennyroyal may have been used as a local anesthetic in ancient Jewish medical practice [118]. Giles of Santarém advised applying a complex mixture of vegetable products, including mint juice, for abscess pain and otalgia. One of these preparations was used as a rubefacient to be applied to persons with leg atrophy, probably as a result of poliomyelitis, demyelinating diseases or even trauma [106]. Thomas Sydenham used liniments containing multiple herbs, including mints, for local applications to the abdomen and armpits in patients with 'scrophular diseases', which probably refer to cervical tuberculous lymphadenitis and rickets [47].

Odo de Magdunensis mentions a mixture of mint, strong rue, tansy, and milk cooked in olive oil with virgin wax, made into a plaster and applied to the kidney area of women who could not deliver their child. This could be to relieve pain [83]. Still on the subject of ophthalmic afflictions, William Mackenzie (1791–1868), author of the *Practical Treatise of the Diseases of the Eye*, one of the first British textbooks of ophthalmology, used a formulation of peppermint water, camphorated spirits of opium tincture and borax as a local anesthetic to be applied to the lacrimal puncta prior to mechanical unblocking with a hair pencil [119].

Gout is an inflammatory arthropathy characterized by the painful swelling of joints resulting from the precipitation of uric acid crystals. Mints are among the several types of treatment tried for this disease throughout history. Pliny exalted the usefulness of pennyroyal for treating gout, presumably by covering the affected body region with the herb [17]. In the work of Hildegard von Bingen also mentioned the usefulness of mints for treating gout [39]. Sydenham, having himself been afflicted with gout in the last years of his life, made very accurate descriptions of the disease and included mints in several electuaries (i.e., medicines, generally in powder, mixed in with a palatable medium) form to be taken by patients, as well as for rheumatism [47]. The anti-gout effect of mints may have a scientific basis as recent studies have shown that mint extracts inhibit xanthine oxidase *in vitro*, an enzyme involved in the formation of uric acid, as well as in generating oxidative stress that contributes to the pathophysiology of the disease [120]. The local anesthetic effect of mints is attributed mainly to volatile monoterpene menthol which, at low concentrations, activates receptors on cold nervous fibers by creating a perception of coolness, whereas it is irritating at high concentrations [121,122].

2.9. For Oral Health

The first mention of mint herbs for oral health dates back to a 4th century Egyptian papyrus, probably written by a Christian monk, on which a recipe for toothpaste appeared. This toothpaste was a mixture of 'one drachma of rock salt, two drachmas of mint, one drachma of dried iris flower and 20 grains of pepper' [123]. Abu Al Qasim Al Zahrawi (Albucasis, 936–1013) recommended washing or gargling with mint decoctions to help with the 'swelling and erosion of the mouth, tongue and throat' brought up by the toxic effects of topically applied mercury, a metal already used in Islamic medicine for its therapeutic value [124]. He also advised inhaling the vapors of pennyroyal fumigation in absinthe and vinegar for a swollen uvula [125]. He added mint to borax for oral hygiene purpose [126]. Nikolaos Myrepsos (fl. 13th century), a physician at the court of John III Doukas Vatatzes at Nicaea wrote in his *Dynameron*, one of the richest treatises of the late Byzantine era, about the virtues of mints for the inflammation of teeth, mouth, and palate [127].

Gilbertus Anglicus (Gilbert of England, 1180–1250) wrote *Compendium Medicinae* (Compendium of Medicine), an important medical treatise of the 13th century, which mentions mints as promoters of oral health. For halitosis caused by teeth or gum decay, Gilbertus advised a mouthwash made from birch and mint soaked in wine, after which a linen cloth would be rubbed against gums until they hemorrhaged. Finally, the patient should chew marjoram, oregano, mint, and pellitory leaves, as well as rub the mixture into gums [128]. Paulus Aegineta wrote that 'Pseudo-Dioscorides recommends mint triturated with honey, red sumach, and rose oil with honey, or by itself for cleaning the tongue' [31]. Guy de Chauliac (1300–1368), the famous surgeon and pioneer in odontology, advised in his magnum opus *Chirurgia Magna* (Great Surgery) using wine and mint mouthwashes for discolored teeth. For carious teeth, Chauliac advocated an antiseptic gargle made of wine mixed with mint, sage, and pepper or pellitory [129]. For odontalgia, Rev. Townsend references one of Boerhaave's own prescriptions for a pill to be applied to the decaying tooth composed of opium, camphor, oil of cloves, and peppermint essential oil [50]. Despite the many recipes found for oral health that included mints, obviously the primary therapeutic intention was to create a local anesthetic/anti-inflammatory effect on the affected site.

2.10. For Cutaneous Disorders

A few references are made about using these herbs for dermatological purposes. One famous effect, apparently to have been discovered by chance, refers to the treatment of elephantiasis. According to Pliny, elephantiasis was prevalent while Gnaeus Pompeius Magnus (Pompey the Great, 106 BC–48 BC) governed and had been imported from Egypt. He wrote that a person, ashamed of being affected with a facial form of the disease, smeared watermint on his face with shame and was cured [17]. A similar account comes from Paulus Aegineta, who mentions a mixture of 'watermint, juniper and mezereon', previously used by Marcellus Empiricus (fl. 385) and Quintus Serenus Sammonicus (d. 212), both medical writers from the late Roman empire [31]. The latter described remedies for different skin problems, such as 'juice of the bark of the juniper, the ashes and blood of the weasel, mint' [31]. Paulus Aegineta advised 'washing the head frequently with a lotion made from marjoram, mint or centaury' for porrago favosa (i.e., favus, a dermatophytosis) [31]. Theophanes Chryssobalantes (fl.c. 950), chief physician of the educated Emperor Constantine VII the Porphirogenitus, in his book *Epitome*, quotes a preparation of the ancient physician Archigenes (1st century AD) that consisted of laudanum and mint in equal quantities for alopecia [130]. Theophanes also indicates a concoction made of celandine (swallow-wort), Egyptian rose, and mint to dye the hair blonde [130].

In *Trotula*, Trota advises anointing *Theriaca magna Galeni* or 'juice of mint' to treat the thickness of lips [40]. William Augustus Hardaway (1850–1923) in his *Manual of Skin Diseases* mentions using peppermint essential oil in a formula for ameliorating generalized pruritus in urticaria and papular eczema, which included carbolic acid, glycerin and water, which would then be sprayed onto skin with an atomizer [131]. Recent research shows that several mint-based products appear promising to treat dermatological conditions. Peppermint essential oil is effective for controlling pruritus and for relieving irritation and inflammation [132,133].

2.11. For Nervous Disorders

Since ancient times, mint-based preparations have also been known to affect cognition and emotion, and have been used as restorative agents to enable to regain vigor. The oldest record on the effect that mints have on the central nervous system is attributed to Aretaeus of Cappadocia (fl. 2nd century), who advised that epileptics should take walks among acrid and aromatic herbs, such as mint and pennyroyal [134]. Pliny also wrote about the use of watermint to control epileptic seizure, besides it helping with hangovers [17]. For the latter, Marcus Terentius Varro (116 BC–27 BC), a roman scholar and writer, advised hanging garlands of pennyroyal in one's chambers [17]. In contrast, in the *Hippocratic Corpus* it is written that mint should be avoided by epileptics because of its 'pungent nature' [135] There are also several records about using mints to treat headaches. Paulus Aegineta treated throbbing headaches and those resulting from heat exposure with pennyroyal and watermint [33]. Galen wrote about a head compress, previously recorded by Asklepiades, but probably attributed to Nikomedes IV of Bithunia, which contained 'sulfurwort (hog-fennel), rue, mint and other herbs in rose oil' [31]. Finally, some attempts have been made to use mints for psychiatric disorders. Galen also wrote about hysteria, and, among his treatments, he administered a mixture of plants, such as mint, hellebore, laudanum, belladonna extract, and valerian [136]. In Trota's work, *Potio Sancti Pauli*, which included horsemint, was given to treat several nervous states, including 'epileptics, cataleptics and analeptics' [40]. Centuries later, Rev. Townsend tried peppermint essential oil, without much success, to treat hysterical fits [50], as did John Quincy (d. 1722), the English apothecary and medical writer, with pennyroyal water [137].

Table 1. Probable medical uses given to different *Mentha* species by the main authors discussed in this review throughout history in chronological order.

Author	*Mentha* Species	Probable Medical Use
Nicander of Colophon (197 BC–170 BC)	Unknown	Snake repellant
Marcus Terentius Varro (116 BC–27 BC)	Pennyroyal	Anticonvulsant?
Pliny (23-79)	Pennyroyal	Stimulate parturition Abortifacient Insect repellant Treatment of snake, scolopendra, and scorpion bites Diuretic Local anesthetic/anti-inflammatory
	Watermint	Treatment of snake, scolopendra, and scorpion bites Anticonvulsant?
	Unknown	Anti-emetic Antiparasitic Anti-inflammatory
Celsus (c. 25 BC–c. 50)	Spearmint	Diuretic
Dioscorides (40–90)	Pennyroyal	Stimulate parturition
	Spearmint	Antiparasitic
Soranus of Ephesus (98–138)	Pennyroyal	Female hygiene
Galen (129–c. 210)	Pennyroyal	Stimulate menses
	Watermint	
	Unknown	Local anesthetic/anti-inflammatory Antidepressant/antipsychotic?
Quintus Serenus Sammonicus (d. 212)	Pennyroyal	Abortifacient
Theodorus Priscianus (b. 300)	Pennyroyal	Local anesthetic/bronchodilator/antitussive?
Aëtios of Amida (502–575)	Pennyroyal	Gastrointestinal (to Improve digestion)
		Antitussive/expectorant
Alexander of Tralles (525–605)	Watermint	Antiparasitic
Paulus Aegineta (625–690)	Pennyroyal	Reptile repellant Anti-inflammatory/analgesic
	Watermint	Anti-inflammatory/analgesic
	Unknown	Improve digestion Carminative Antiparasitic Antifungic Flavorant
Abulcassis (936–1013)	Pennyroyal	Local anesthetic/anti-inflammatory
	Unknown	
Theophanes Chryssobalantes (fl.c. 950)	Unknown	Treatment for alopecia
Avicenna (980–1037)	Watermint	Spermicide Reduce libido
Odo de Magdunensis (1070?–1112?)	Unknown	Antiparasitic Local anesthetic

Table 1. *Cont.*

Author	*Mentha* Species	Probable Medical Use
Hildegard von Bingen (1098–1179)	Pennyroyal	Stimulate parturition
	Spearmint	Improve digestion
	Watermint	Improve digestion Reduce libido
	Unknown	Local anesthetic/anti-inflammatory
Gilbertus Anglicus (1180–1250)	Unknown	Local anesthetic/anti-inflammatory
Giles of Santarém (1185–1265)	Unknown	Local anesthetic/anti-inflammatory
Trota (fl. 12th century)	Horsemint	Carminative Insect repellant? Diuretic? Anticonvulsant/antidepressant/neuroleptic?
	Pennyroyal	Induce receding of uterine prolapse Increase fertility Diuretic?
	Unknown	Stimulate menses and parturition Reduce libido Anti-inflammatory?
Nikolaos Myrepsos (fl. 13th century)	Unknown	Anti-inflammatory
John of Gaddesden (1280–1361)	Watermint	Female hygiene
Guy de Chauliac (1300–1368)	Unknown	Oral hygiene
Paracelsus (1493–1541)	Unknown	Improve digestion
Garcia de Orta (1501–1568)	Unknown	Anti-emetic
Cristóvão da Costa (1515–1594)	Unknown	Anti-emetic
Luis de Mercado (1525–1611)	Pennyroyal	Stimulate menses
Prosper Alpinus (1553–1617)	Unknown	Aanti-emetic
Robert Burton (1577–1640)	Watermint	Carminative
James Primerose (d. 1659)	Pennyroyal	Abortifacient
Thomas Willis (1621–1675)	Unknown	Anti-emetic Carminative
Thomas Sydenham (1624–1689)	Watermint	Spasmolytic
	Unknown	Local anesthetic/anti-inflammatory Anti-emetic Antiparasitic
Sarah Jinner (fl. 1658–1664)	Pennyroyal	Abortifacient, stimulate menses
Herman Boerhaave (1668–1738)	Peppermint	Anti-emetic Local anesthetic Antidepressant/antipsychotic?
	Watermint	Anti-emetic
John Quincy (d. 1722)	Pennyroyal	Antidepressant/antipsychotic?
Reverend Joseph Townsend (1739–1816)	Peppermint	Local anesthetic Spasmolytic Antidepressant/antipsychotic?

Table 1. *Cont.*

Author	*Mentha* Species	Probable Medical Use
Jean-Paul Marat (1743–1793)	Unknown	Antitussive/antibiotic?
William Currie (1754–1828)	Peppermint	Improve digestion Spasmolytic Antiparasitic
William Mackenzie (1791–1868)	Peppermint	Local anesthetic
Albert Ethelbert Ebert (1840–1906)	Peppermint	Flavorant
William Augustus Hardaway (1850–1923)	Peppermint	Local anesthetic/anti-inflammatory
Isabel Robb (1860–1910)	Peppermint	Spasmolytic

Table 2. Description and main results of relevant studies assessing the health-promoting effects of mint-based products.

Authors	Mint-Based Product	Study Type	Species	Main Biological Effect
Innamori et al. (2017) [52]	Peppermint oil	*In vivo*	Healthy human subjects	Acceleration of gastric emptying
Hills and Aaronson (1991) [53]		*In vivo*	Guinea pigs	Relaxation of taenia coli
Zong et al. (2011) [54]		*In vivo*	Sprague-Dawley rats	Stimulation of bile secretion
Anderson and Gross (2004) [55]		*In vivo*	Human subjects (ambulatory surgery patients)	Reduction of post-operative nausea
Tayarani-Najaran et al. (2013) [56]	Spearmint and peppermint oils	*In vivo*	Human subjects (patients undergoing chemotherapy)	Reduction of chemotherapy-induced nausea and vomiting
Asao et al. (2001) [57]	Peppermint oil	*In vivo*	Human subjects (undergoing colonoscopy)	Reduction of colonic spasm
Maggiore et al. (2012) [68]	Pennyroyal and peppermint oils	*In vitro*	*Equinococcus granulosus* (protoscoleces)	Protoscolicidal effect
Katiki et al. (2011) [69]	Peppermint oil	*In vitro*	*Haemonchus contortus* and *Trichostrogylus* spp.	Anthelminthic activity
Girme et al. (2006) [70]	Peppermint extract	*In vitro*	*Pheritima posthuma*	Anthelminthic activity
Akdogan et al. (2004) [93]	Spearmint and peppermint teas	*In vivo*	Male Wistar rats	Decrease in total plasma testosterone levels Arrest of spermatogenesis
Ansari et al. (2000) [94]	Peppermint oil	*In vitro*	*Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* (IIIrd instar larvae)	Larvicidal activity
		In vivo		Repellant activity when applied in human skin
Erler et al. (2006) [95]	Peppermint oil	*In vitro*	Female *Culex pipiens*	Repellant activity
Rocha et al. (2015) [97]	Pennyroyal oil	*In vitro*	*Anopheles atroparvus*, *Anopheles gambiae*, *Anopheles stephensi* and *Aedes aegypti*	Larvicidal activity
Eccles et al. (1990) [108]	Menthol lozenge	*In vivo*	Human subjects	Subjective sensation of nasal decongestion
Sharma et al. (2018) [109]	Wildmint oil	*In vivo*	Guinea pigs	Relaxation of bronchial smooth muscle and suppression of immunological response to ovalbumin
Laude et al. (1994) [110]	Menthol	*In vivo*	Guinea pigs	Antitussive (reduction of cough frequency)
Paul et al. (2010) [111]	Vicks VapoRub	*In vivo*	Human subjects (children with symptoms of upper respiratory tract infections)	Symptomatic relief of nocturnal cough, congestion, and sleep difficulty
Shahid et al. (2018) [113]	Menthol	*In vitro*	RAW 264.7 cell line	Suppression of lipopolysaccharide-stimulated cytokine release
Lahlou et al. (200) [115]	Hairy mint (*Mentha X villosa var. alopecuroides* Hull) oil	*In vivo*	Male Wistar rats (under anesthesia)	Hypotension and bradycardia
Elsaie et al. (2016) [132]	Peppermint oil	*In vivo*	Human subjects with chronic pruritus	Improvement of pruritus

3. Conclusions

This paper makes a thorough descriptive review of the many medical uses given to mints throughout history, from ancient civilizations to modern day medicine, beyond previously published material, highlighting both the authors in medical culture responsible for their dissemination, as well as their major galenic formulations. Most medical knowledge came from Ancient Greek and Roman philosophers and medical authors, while later contributions consisted mainly of progressive technological improvements. From the immediately perceived qualities of these herbs, it can be inferred that the primary intention of their topical application was to probably cause local anesthesia and to control irritation and inflammation, being especially used in gastrointestinal tract affections. Their particular scent and flavor also came of use for the purpose of masking the unpleasant taste of many medicinal formulae long before the advent of pharmaceutical technology. The longevity and diversity of the use of mints in medicine are a testament of their importance, receiving still noble place in herbal medicine.

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Article

Wound Healing Effects of *Aloe muth-muth*: In Vitro Investigations Using Immortalized Human Keratinocytes (HaCaT)

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Simple Summary: *Aloe muth-muth* is a cross-bred species cultivated from the well-known Aloe plants, namely *Aloe vera* and *Aloe ferox* through forced pollination. Considering that Aloe plants were traditionally widely used for treatment of wounds and skin lesions, *Aloe muth-muth* was also thought to have possible wound healing properties. Therefore, this study tested the ability of parts from the *Aloe muth-muth* plant to improve the closure of wounds induced in human skin cell layers grown in an incubator in a laboratory. Both the whole leave material and the inner gel-like part of this plant were tested for wound healing properties. It was found that the *Aloe muth-muth* gel part taken from the leaves possesses very high wound healing activity and can possibly be used in future wound treatments.

Abstract: The traditional use of *Aloe* spp. for the purpose of wound healing has a long history and is widespread internationally. Recently, a hybrid aloe plant (*Aloe muth-muth*) has been cultivated by cross pollination between *Aloe vera* and *Aloe ferox*. The *Aloe muth-muth* plant has not yet been investigated for medicinal properties and provides an opportunity for potential biological activity, including wound healing. The aim of this study was to investigate the in vitro wound healing effects of both *Aloe muth-muth* gel and whole leaf material with the use of the immortalized human keratinocyte (HaCaT) cell line. Cell viability was conducted using methyl thiazolyl tetrazolium (MTT) assays. In vitro wound healing was tested on HaCaT cells using an established scratch assay method. The effect of *Aloe muth-muth* gel material on HaCaT cell migration was also investigated. *Aloe muth-muth* gel material exhibited statistically significantly ($p < 0.05$) higher percentage wound closure compared to the control at all three concentrations investigated. These findings confirm that this newly cultivated species, *Aloe muth-muth*, also possesses wound healing activity corresponding to that reported for the two species it is derived from, namely, *Aloe vera* and *Aloe ferox*. Therefore, *Aloe muth-muth* has the potential to be used in future wound therapeutics.

Keywords: *Aloe muth-muth*; cell migration; HaCaT cells; in vitro cytotoxicity; scratch assay; wound healing

1. Introduction

A wide variety of products, substances, and dosage forms, such as alginates, antimicrobials, foams, and hydrocolloids, amongst others, are used in the treatment of wounds [1]. Phytochemicals that

are found in traditional wound healing remedies commonly have antioxidant or anti-inflammatory properties [2,3]. Plants of the *Terminalia* genus (e.g., turmeric) are examples of such remedies used in Asian traditional wound healing remedies [2,4]. Other plant species that have been investigated for wound healing include *Anagallis arvensis* L. and *Anagallis foemina* Mill., for which anti-inflammatory and bacteriostatic properties (in vitro) have been reported [5]. Similarly, *Bulbine frutescens* and *Bulbine natalensis* have been found to exhibit significant improvement in wound contraction compared to an untreated control in animal studies [6]. The use of *Aloe* plant material as a wound healing remedy is especially notable and has been described, by Dioscorides, as early as the first century A.D. [7,8]. The wound healing effects of materials from different *Aloe* species have been investigated scientifically, both in vitro and in vivo. Increased wound area reduction was reported in mice treated with *Aloe vera* powder, in combination with poly(lactic-co-glycolic acid) (PLGA) nanofiber dressings and recombinant human epidermal growth factor (rhEFG), in comparison with controls of PLGA and rhEGF only [9]. *Aloe vera*, *Aloe ferox*, and *Aloe marlothii* leaf materials (gel and whole leaf extracts) have demonstrated increased wound healing in a zone exclusion-type assay using immortalized human keratinocytes (HaCaT cells) [10]. The in vitro wound healing potential of *Aloe vera* has also been demonstrated using human primary epidermal keratinocytes (HPEK) and a human skin equivalent model, with increased expression of integrin receptors (β1, α6, and β4) and E-cadherin being observed in HPEK treated with *Aloe vera* [11]. *Aloe muth-muth* has recently been cultivated by means of forced cross-pollination between *Aloe vera* and *Aloe ferox* at Rooiklip nursery in Swellendam, South Africa. It has thorny leaves that resemble those of *Aloe vera*, but also features erect racemes of yellow flowers. However, the medicinal properties such as the wound healing potential of *Aloe muth-muth* is still unknown.

Consequently, this study aimed to determine the cytotoxicity, wound healing, and migratory activity of *Aloe muth-muth* gel and whole leaf material using the human keratinocyte (HaCaT) cell line.

2. Materials and Methods

2.1. Preparation of Aloe Muth-Muth Whole Leaf and Gel Materials

Leaves of *Aloe muth-muth* plants were provided by Mr. Jaap and Hannes Viljoen of Rooiklip Nursery in Swellendam, South Africa. A voucher was deposited at the North-West University herbarium with accession number PUC0014886. The inner gel material and outer rind were separated by manually filleting the leaves with knives [10]. This filleting process basically entails cutting off the leaf base and tapering point, then peeling off the outer rind from the top and bottom sides, leaving an inner gel-like fillet. After rinsing with water, the inner gel material was liquidized in a kitchen blender and the outer rind parts were pulverized using a Retsch MM400 mixer mill (Retsch GmbH, Haan, Germany). To prepare *Aloe muth-muth* whole-leaf material, we added a quantity of the rind to the gel material in a 1:1 ratio that reflects the approximate ratio of gel to rind in a real *Aloe* leaf. Separate powdered materials were obtained by freezing the gel material and the whole leaf at −80 °C, followed by lyophilization with a VirTis freeze-dryer (Winchester, United Kingdom) until completely dry.

2.2. Characterization of Aloe Muth-Muth Whole Leaf and Gel Materials

The *Aloe muth-muth* gel and whole leaf plant materials were chemically characterized with quantitative proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), and spectra were obtained with a Bruker Avance III HD NMR (Bruker Corporation, Billerica, MA, USA). The quantities of marker molecules (i.e., aloverose, glucose, malic acid, lactic acid, citric acid, and whole leaf marker) in the *Aloe muth-muth* gel and whole leaf materials were determined according to a previously published method [12].

2.3. Culturing of HaCaT Cells

HaCaT cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Separations, Johannesburg, South Africa), supplemented with 10% fetal bovine serum (FBS)

(ThermoFisher Scientific, Johannesburg, South Africa), 1% penicillin/streptomycin (10,000 U/mL, Lonza, Whitehead Scientific (Pty) Ltd., Cape Town, South Africa), 1% non-essential amino acids (NEAA) (Lonza), and 2 mM L-glutamine (Lonza). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2 using an ESCO CelCulture CO_2 incubator (ESCO Technologies Inc., Horsham, PA, USA). Cells were cultured in T75 cm^2 flasks. Growth medium was changed every 48 to 72 h and cells were viewed under a Nikon TS100 light microscope (Nikon Instruments, Tokyo, Japan) in order to estimate confluence.

2.4. Sub-Culturing of HaCaT Cells

HaCaT cells were sub-cultured by trypsinization at 70% to 80% confluency. Spent growth medium was removed from the culture flasks and the cells were rinsed twice with 10 mL phosphate-buffered saline (PBS; HyClone) to remove any residual medium. After we added 3 mL trypsin-ethylenediaminetetraacetic acid (EDTA) (Lonza), the flasks were incubated for 9-12 min at 37 °C. To neutralize the trypsin, we added 6 mL preheated growth medium to the flasks and washed the mixture thoroughly to remove all the cells from the flask surface. The cell suspensions were centrifuged at 140× *g* for 5 min and the supernatant was removed without disturbing the cell pellets. The pellets were then resuspended in growth medium and the suspension was divided using a 1:15 to 1:20 ratio into new flasks. Preheated growth medium was then added to the flasks to a final volume of 15 mL and the flasks returned to the incubator.

2.5. Methyl Thiazolyl Tetrazolium (MTT) Cell Viability Assay

Viability was measured after 24 and 48 h with the MTT assay [13] in order to evaluate the cytotoxicity of the plant materials at various concentrations. The experimental groups consisted of three concentrations of *Aloe muth-muth* gel or whole leaf (0.4, 0.6, and 1.3 mg/mL) [10]. In addition to the experimental groups, we included a dead cell control (treated with Triton X-100) and an untreated control, as well as a dimethyl sulfoxide (DMSO) blank group of wells. All experimental groups were tested in triplicate, whereas the control groups were tested in six replicates.

Cells were cultured to 80% confluence, trypsinized, and had their viable cell count determined using trypan blue exclusion. Cells were seeded in 96-well plates at 125,000 cells/mL (200 µL/well) and incubated for 24 h. After the 24 h incubation, the medium of the experimental groups were removed and replaced with medium containing plant material added at the relevant experimental concentrations. The experimental concentrations were obtained by preparing stock solutions with supplemented DMEM and then further diluting the stock solution to the relevant experimental concentration. Prior to dilution, the stock solutions were filtered using a 0.45 µm syringe filter. Control groups received medium without added plant material. The plates were then incubated.

At time point 48 h, after we added the experimental solutions to the HaCaT cells, the medium in all experimental groups and the untreated control group was aspirated and the cells were washed twice with 100 µL PBS. The dead cell control group was treated with Triton X-100 (0.2% in PBS) that was removed after 15 min. A volume of 180 µL serum and additive-free DMEM along with 20 µL MTT solution (5 mg/mL stock solution in PBS) was added to the experimental and control groups. The plates were then covered with aluminum foil and incubated for 90 min. After the incubation period, we added 200 µL DMSO to every experimental, control, and DMSO blank well to dissolve the formazan crystals that formed during incubation. The plates were consequently placed on an orbital shaker for 1 h to dissolve the formazan completely. After 1 h, the absorbance was measured at 560 nm and 630 nm.

Cell viability was consequently calculated using the following equation (Equation (1)):

$$\% \text{ Cell viability} = (\Delta\text{Sample} - \Delta\text{Blank})/(\Delta\text{Control} - \Delta\text{Blank}) \times 100 \quad (1)$$

where

ΔSample = absorbance of treated cells$_{560}$ − absorbance of treated cells$_{630}$;
ΔBlank = mean absorbance of blank$_{560}$ − mean absorbance of blank$_{630}$;
ΔControl = mean absorbance of untreated control$_{560}$ − mean absorbance of untreated control$_{630}$.

2.6. In Vitro Wound Healing Scratch Assay

HaCaT cells were trypsinized at 80% confluence and counted with trypan blue using a hemocytometer (Marienfield-Superior, 0.0025 mm^2, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). A cell suspension was prepared, and cells were seeded at a density of 400,000 cells/mL. A volume of 2.5 mL of this prepared cell suspension was seeded into every well of a 12-well plate (Corning Costar Corporation, Corning, NY, USA). The plate was then incubated at 37 °C in a 5% CO_2 humidified atmosphere for 24 h. After 24 h, the cells were visualized under a microscope to establish the formation of a monolayer in each well. Experimental solutions of the selected plant materials were prepared by preparing a stock solution of plant material in DMEM growth medium and diluting to appropriate concentrations (0.4, 0.6, and 1.3 mg/mL for *Aloe muth-muth* gel and whole leaf). Both *Aloe muth-muth* gel and whole leaf materials were tested for wound healing. Scratches were induced in the monolayers across the diameter of the wells using a sterile 200 µL pipet-tip [14,15]. The culture medium in each well was aspirated and each well was washed 4 times with serum- and additive-free DMEM. A volume of 4 mL of each concentration, as well as culturing medium only (untreated control), was added to the wells in triplicate. Photos of each well were taken immediately after scratches were induced and at 8 h intervals, thereafter, for a total period of 48 h. The photos were taken with a camera (The Imaging Source DFK 72AUC02) mounted on a Nikon TS100 light microscope (Nikon Instruments, Tokyo, Japan). For the duration of the experiments, we incubated the plates in a humidified environment with 5% CO_2 at 37 °C. ImageJ software was used to measure the wound surface area, and the percentage wound closure was calculated according to the following modified equation [10]:

$$\% \text{ Wound closure} = [((\text{Pre-migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}})/ (\text{Pre-migration})_{\text{surface area}}] \times 100 \quad (2)$$

where $(\text{Pre-migration})_{\text{surface area}}$ is the initial wound surface area (µm^2) at time 0 h, and $(\text{Migration})_{\text{surface area}}$ is the wound surface area (µm^2) at a specific time point. The closure rate (µm^2/h) was calculated with the following modified equation [10]:

$$\text{Closure rate } (\mu m^2/h) = [((\text{Pre-migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}})/\text{Time (h)}] \quad (3)$$

2.7. In Vitro Cell Migration Assay

The effects of *Aloe muth-muth* gel (selected on the basis of the wound closure results) on cell migration was evaluated using the Cell Biolabs CytoSelect 24-Well Cell Migration Assay kit. The kit consisted of a 24-well cell culture plate with 12 polycarbonate membrane inserts (8 µm pore size). Individual cell suspensions containing 1×10^6 cells/mL were prepared, having been resuspended in serum- and additive-free DMEM after sub-culturing. The suspensions were centrifuged at 140× *g* for 5 min. Each individual pellet was then resuspended in 1 mL serum- and additive-free DMEM containing the same working concentrations of *Aloe muth-muth* gel as used in the scratch assay (0.4, 0.6, and 1.3 mg/mL). The untreated control was resuspended in serum- and additive-free DMEM only. A volume of 500 µL of DMEM growth medium was added to each bottom well (outside the inserts). A volume of 300 µL of each prepared suspension containing experimental concentrations, as well as 300 µL of the untreated control, were added to the inside of the inserts in triplicate. The plates were then incubated in a humidified environment with 5% CO_2 for 24 h. After the incubation period, we aspirated the media in each insert, and wetted cotton-tipped swabs were used to gently clean out the interior of each insert. Each insert was then transferred to a clean well containing 400 µL of the

supplied staining solution and incubated for 10 min at room temperature. The inserts were gently rinsed using a beaker of water and allowed to air dry. Each insert was then added to another clean well containing 200 µL of the supplied extraction solution and incubated for 10 min on an orbital shaker. A volume of 100 µL of each sample was added to a 96-well microtiter plate and the absorbance was measured at 560 nm.

2.8. Statistical Analysis

All the experiments were performed in triplicate and the data were statistically analyzed with STATISTICA Version 12 (Statsoft, Tulsa, OK, USA). A one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test were performed, with statistically significant differences accepted at $p < 0.05$. The non-parametric Kruskal–Wallis test was used as verification. For the MTT assay results, we used the Bonferroni test to assess the statistically significant differences between each treatment and the untreated control (significance accepted at $p < 0.05$). The data were presented as mean ($n = 3$) ± standard deviation (error bars).

3. Results and Discussion

3.1. Characterization of Aloe Muth-Muth Whole Leaf and Gel Materials

The ^{1}H-NMR spectra for the gel and whole leaf materials are shown in Figure 1a,b, respectively. The content of marker molecules in the *Aloe muth-muth* gel and whole leaf materials obtained from quantitative ^{1}H-NMR are shown in Table 1.

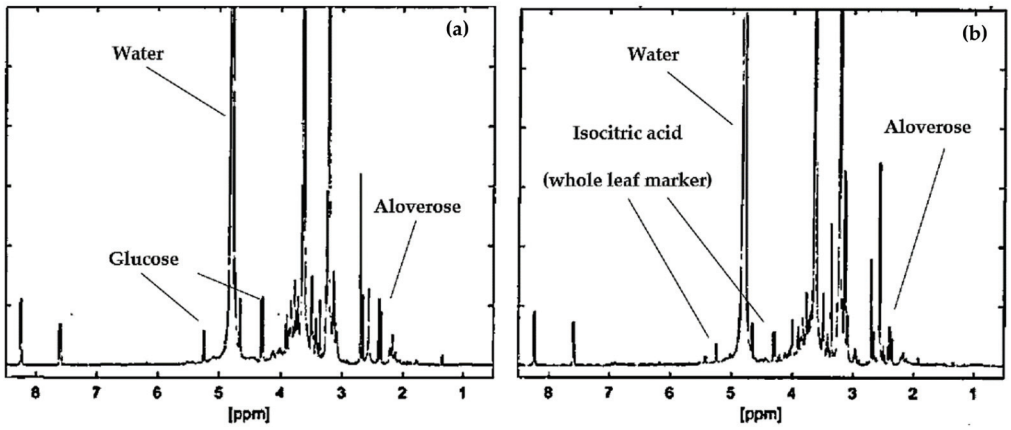

Figure 1. Quantitative proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) spectra for *Aloe muth-muth* gel (**a**) and *Aloe muth-muth* whole leaf (**b**) materials.

Table 1. Quantity of marker molecules in *Aloe muth-muth* gel and whole leaf materials determined by ^{1}H-NMR spectroscopy.

Component	*Aloe muth-muth* Gel		*Aloe muth-muth* Whole Leaf	
	Content (%)	Content (mg/mL)	Content (%)	Content (mg/mL)
Aloverose (polysaccharide)	11.3	793.8	8.1	568.3
Glucose	11.7	821.1	6.8	477.2
Malic acid	10.4	730.8	5.4	380.3
Lactic acid	0.1	12.5	Traces	ND
Citric acid	ND	ND	1.5	103.8
Iso-citric acid (whole leaf marker)	ND	ND	5.1	355.8

Key to abbreviations: ND—not detected.

As expected, the composition as listed in Table 1 and Figure 1 for *Aloe muth-muth* is similar to that of *Aloe vera* and *Aloe ferox* with respect to the type and quantity of marker chemical compounds present [10].

3.2. Cytotoxicity Testing of Aloe Muth-Muth Gel and Whole Leaf Material Using the MTT Assay

The MTT assay results for *Aloe muth-muth* gel and whole leaf materials after 48 h exposure are shown in Figure 2a,b, respectively. It is evident from the data depicted in Figure 2 that the *Aloe muth-muth* gel and whole leaf materials showed a concentration-dependent decrease in cell viability, as measured with the MTT assay after 48 h. At 24 h exposure, no significant effects were observed on the cell viability (results not shown). López-García et al. [16] provided guidelines on the cytotoxicity level of a compound on the basis of its effect on the in vitro cell viability percentage. A compound is considered non-cytotoxic when a cell viability of higher than 80% is obtained; weak cytotoxicity is associated with 60–80% cell viability, moderate cytotoxicity is linked with 40–60% viability, and a cell viability lower than 40% indicates strong cytotoxicity. Overall, both the *Aloe muth-muth* gel and whole leaf material displayed weak to non-cytotoxic properties and can therefore be considered safe to use on human skin. This is in line with previous findings for the gel and whole leaf materials of different aloe species [10,17].

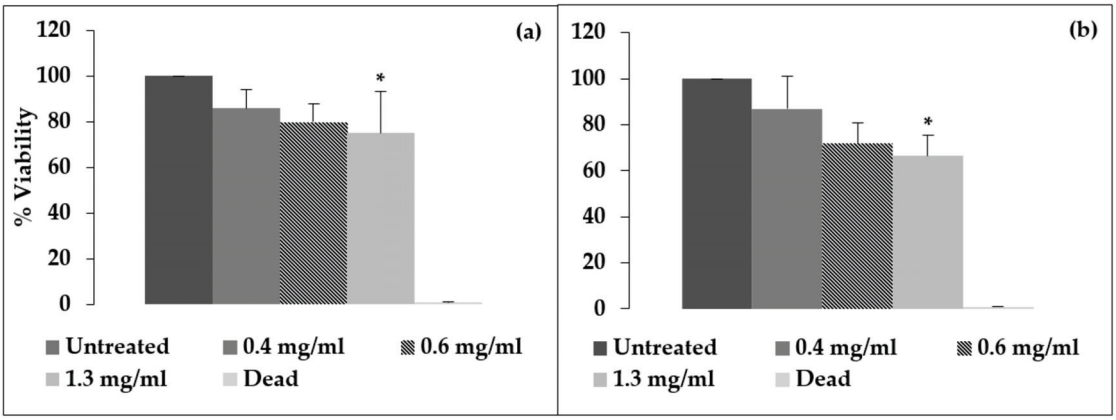

Figure 2. Percentage cell viability of immortalized human keratinocyte (HaCaT) cells after 48 h exposure to *Aloe muth-muth* gel (**a**) and *Aloe muth-muth* whole leaf (**b**) plant material. The data were normalized to the untreated control that is considered as 100% viable. The * indicates statistical significance in comparison to the untreated control (Bonferroni test, $p < 0.05$).

3.3. Measuring the Re-Epithelialization Potential of Aloe Muth-Muth Gel and Whole Leaf Material Using the Scratch Assay

Figure 3 presents the percentage wound closure and migration rate results for *Aloe muth-muth* gel and whole leaf material as a ratio of the untreated control. Microscopic images depicting the wound gap closure results after 24 and 48 h of treatment with *Aloe muth-muth* gel and whole leaf material are depicted in Figures 4 and 5, respectively. It is evident from Figure 4 that the application of *Aloe muth-muth* gel to HaCaT cells with scratched wound gaps resulted in notable improvement in wound gap closure in comparison to the untreated control. The wound closure results in Figure 3a1,a2 indicated the effectiveness of *Aloe muth-muth* gel with respect to wound healing, exhibiting 2-fold to 2.5-fold higher wound closure in comparison to the untreated control.

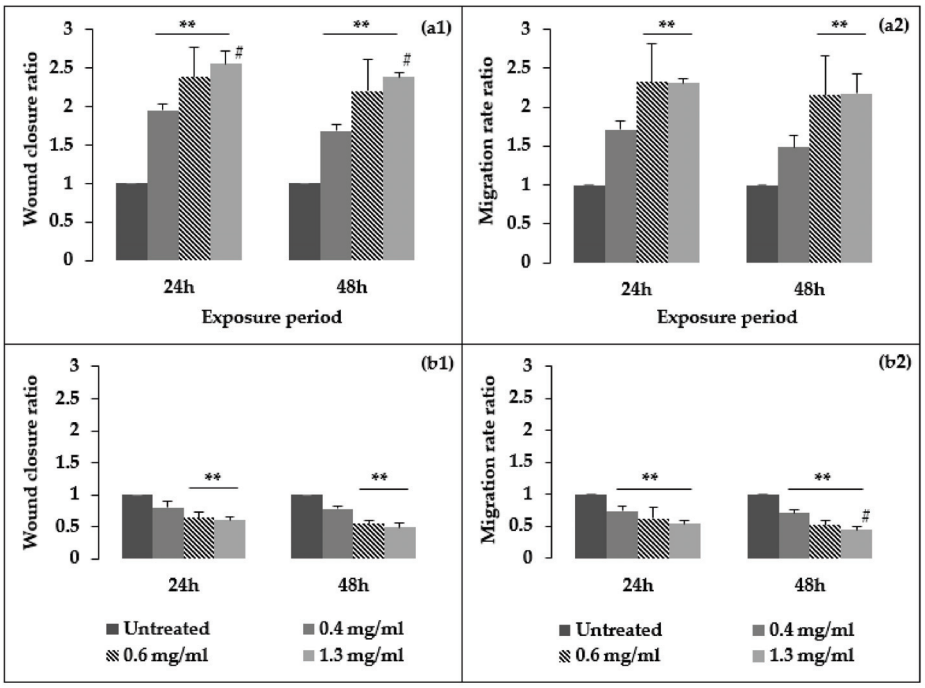

Figure 3. Ratio of percentage wound closure (1) and wound closure rate (2) results after exposure to (**a**) *Aloe muth-muth* gel and (**b**) *Aloe muth-muth* whole leaf at 24 h and 48 h treatment periods. Each data point was calculated with respect to the relevant initial wound value at time 0 h and normalized to the untreated control, which was considered as 1. The ** and # indicate statistical significance determined with Tukey's honest significant difference (HSD) and Kruskal–Wallis tests, respectively, in comparison to the untreated control (significance accepted when $p < 0.05$).

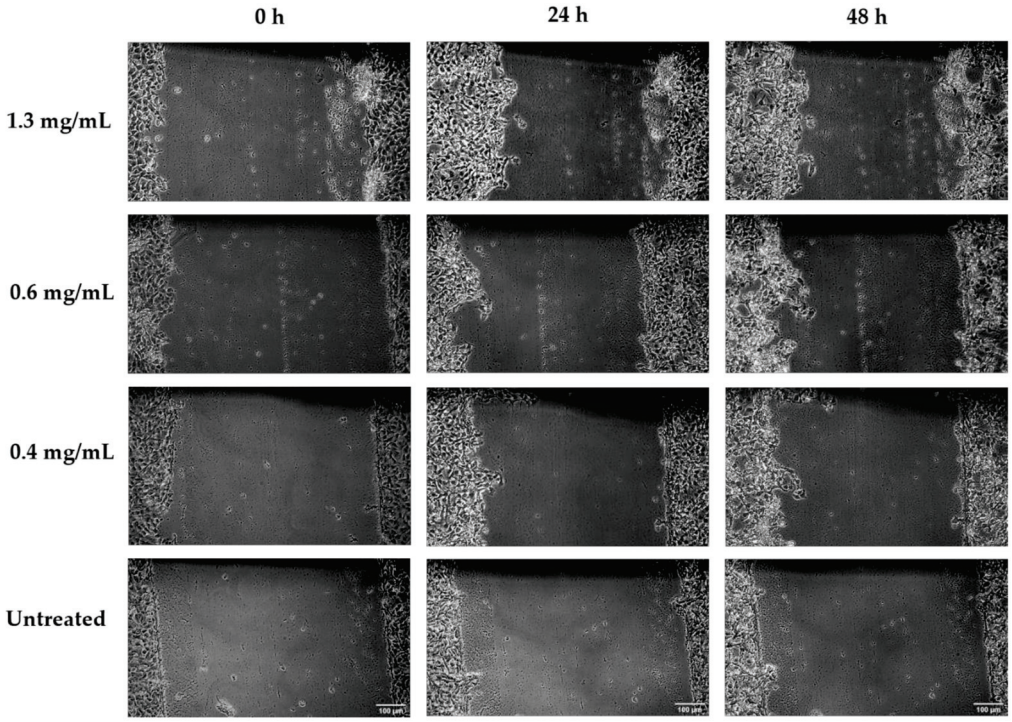

Figure 4. Microscopic photos of wound gaps in HaCaT cells introduced by the scratch technique after treatment with *Aloe muth-muth* gel at 1.3 mg/mL, 0.6 mg/mL, and 0.4 mg/mL, compared to an untreated control, at 0 h, 24 h, and 48 h. Images were captured at 10× magnification and the scale bars indicate 100 µm.

This notable improvement in wound closure, which was statistically significant (Tukey's HSD test, $p < 0.05$), was obtained at all three concentrations of *Aloe muth-muth* gel investigated in this study. The improvement in wound healing for the *Aloe muth-muth* gel material was concentration-dependent, with the highest concentration of 1.3 mg/mL of the gel resulting in the highest percentage wound closure. In accordance with the percentage wound closure caused by the *Aloe muth-muth* gel material, we also found an increase in the closure rate (Figure 3a2) of the wounds in comparison to the untreated control, but only the wound closure rates of the 0.6 and 1.3 mg/mL concentrations were statistically significant (Tukey's HSD test, $p < 0.05$). Several studies have reported *Aloe vera* gel to increase keratinocyte proliferation by upregulating the expression of vascular endothelial growth factor-A (VEGF-A) [18,19]. The major compounds responsible for this phenomenon are aloesin, aloin, and emodin. The gel and whole leaf extracts of different *Aloe* species have also shown the ability to hydrate the skin and reduce erythema, which can be attributed to the polysaccharides such as aloverose that are present in both the gel and whole leaf [20].

In contrast to the promising wound healing results obtained with the *Aloe muth-muth* gel, the *Aloe muth-muth* whole leaf material (Figure 3b1,b2 and Figure 5) did not give similar wound healing results. Both wound gap closure and closure rate were not increased in comparison to the untreated control. This can possibly be attributed to differences in the chemical composition of the whole leaf material compared to the gel material. As shown in Table 1, the whole leaf material contained less aloverose (a bioactive polysaccharide in aloe gel material) than the gel material, but more citric acid and iso-citric acid. These chemical differences were due to the inclusion of leaf rind material in the *Aloe muth-muth* whole leaf material that was not part of the *Aloe muth-muth* gel material. Citric acids have been shown to induce cell-cycle arrest and apoptosis via the caspase- and mitochondrial-dependent signaling pathways in HaCaT cells [21].

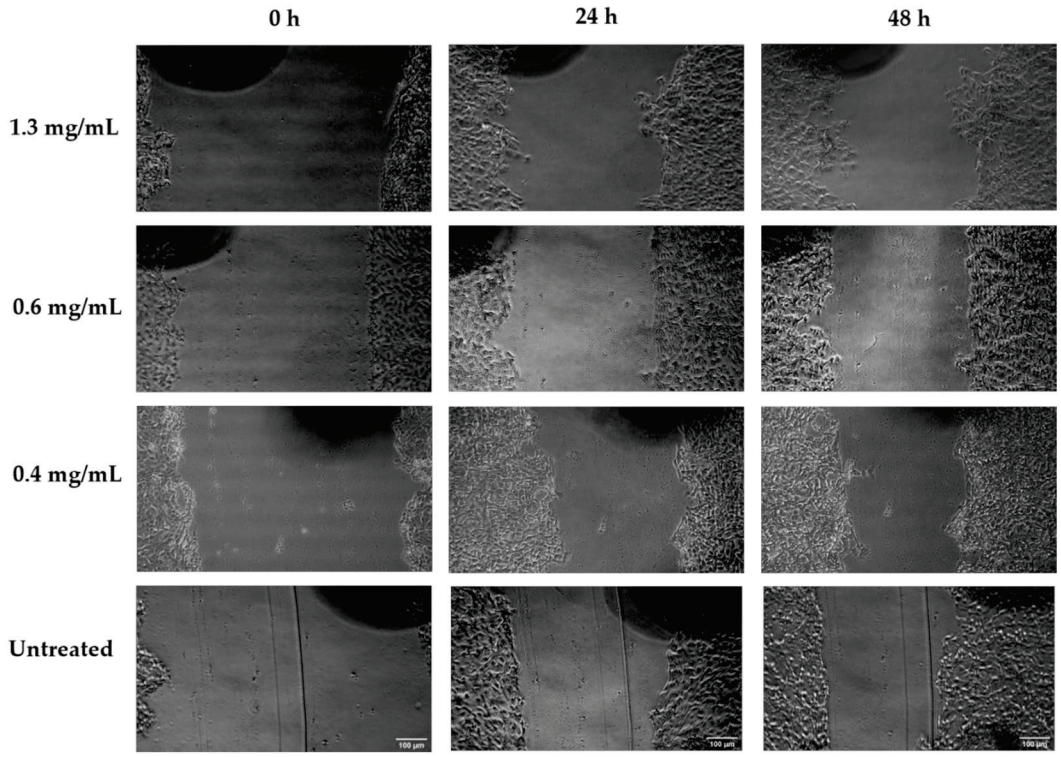

Figure 5. Microscopic photos of wound gaps in HaCaT cells introduced by the scratch technique after treatment with *Aloe muth-muth* whole leaf material at 1.3 mg/mL, 0.6 mg/mL, and 0.4 mg/mL, compared to an untreated control, at 0 h, 24 h, and 48 h. Images were captured at 10× magnification and the scale bars indicate 100 µm.

3.4. Measuring the Migration Enhancement Activity of Aloe muth-muth Gel Using the In Vitro Cell Migration Assay

In general, the lowest concentration (0.4 mg/mL) of the *Aloe muth-muth* gel resulted in a decrease in cell migration compared to the untreated control, while a concentration-dependent improvement in cell migration was observed for the 0.6 and 1.3 mg/mL concentrations (results not shown). The increase in cell migration in comparison to the untreated control was, however, not statistically significant ($p > 0.05$). Therefore, at higher *Aloe muth-muth* gel concentrations, cell migration may play a possible role in the wound healing effect. Aloesin is a compound in *Aloe* species that has been shown to increase cell migration via the phosphorylation of cytokines and growth factors [22].

4. Conclusions

Aloe muth-muth gel and whole leaf materials were investigated for wound healing properties using the scratch assay with the HaCaT cell culture model. MTT assays indicated that none of the plant materials at the concentration ranges used in this study showed cytotoxic effects against HaCaT cells. *Aloe muth-muth* gel exhibited significant wound healing properties as indicated by a statistically significant increase in the percentage wound gap closure and migration rate for the two highest concentrations used in this study, in comparison to an untreated control. *Aloe muth-muth* whole leaf material showed some wound healing effects, but to a much lower extent than the gel material, and this was not statistically significant. In conclusion, gel and whole leaf extract of the newly cultivated species, *Aloe muth-muth,* showed potential as a wound healing therapeutic agent in future drug development strategies, following in the footsteps of the species from which it was cultivated, namely, *Aloe vera* and *Aloe ferox*.

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Conflicts of Interest: The authors declare no conflict of interest.

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Ganoderma Lucidum from Red Mushroom Attenuates Formaldehyde-Induced Liver Damage in Experimental Male Rat Model

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Simple Summary: Formaldehyde exposure is common due to inhalation and its presence in some food additives. Upon exposure to formaldehyde via any route, it is majorly metabolized by the liver. However, this metabolism impacts negatively on the liver, and in certain concentrations can result in liver damage referred to as hepatotoxicity. This toxicity is evident by a decrease in antioxidant markers as well as an increase in liver function enzymes, inflammatory markers as well as lipid profile in Wistar rats as shown by this study. To combat the deleterious effect of formaldehyde exposure, this study has shown that *Ganoderma lucidum* from red mushroom presents an excellent natural resource by ameliorating the aforementioned liver toxicity markers. This study should serve as a deterrent for those in the practice of using formaldehyde as food additives. Environment inspectors and governments should ensure that formaldehyde is kept below its toxicity threshold in work environments. However, in cases where hepatotoxicity has ensued or is suspected, *Ganoderma lucidum* could serve as a way to combat this toxicity but should be used under appropriate medical expert supervision.

Abstract: The majority of liver-related illnesses are caused by occupational and domestic exposure to toxic chemicals like formaldehyde (FA), which is widely common in Africa and the world at large. Hence, measures should be taken to protect humans from its hazardous effects. This study, therefore, examines the protective potential of *Ganoderma lucidum* (100 mg/kg body weight) on formaldehyde-induced (40%) liver oxido-inflammation in male rats. Male Wistar rats, 150–200 g, were allotted into four groups of 10 animals as follows: Group 1 was orally treated with 1 mg/mL distilled water, Group 2 was exposed to a 40% formaldehyde vapor environment for 30 min per day, Group 3 was orally treated with

100 mg/kg ethanol extract of *Ganoderma lucidum*, and Group 4 was co-administered formaldehyde and 100 mg/kg ethanol extract of *Ganoderma lucidum*. Rats were then sacrificed 24 h after administering the last dose of treatment, and the livers were excised. *Ganoderma lucidum* significantly reversed the formaldehyde-mediated reduction in body and organ weight. *Ganoderma lucidum* administration significantly prevented oxido-inflammation by reducing the levels of hydrogen peroxide and malondialdehyde and increasing the activity of antioxidant enzymes and glutathione contents, as well as the normal level of nitrite and myeloperoxidase production in FA-treated rats. Additionally, *Ganoderma lucidum* reversed a large decline in proinflammatory markers in formaldehyde. Furthermore, *Ganoderma lucidum* restores formaldehyde-induced histological alterations in the liver. Collectively, our results provide valuable information on the protective potential of *Ganoderma lucidum* in protecting formaldehyde-induced liver oxido-inflammation in male rats.

Keywords: *Ganoderma lucidum*; formaldehyde; liver; oxidative stress; inflammation

1. Introduction

Occupational and household formaldehyde is a common hydrophilic compound that is immediately retained through the lungs and, to a much lower extent, the skin. Health effects related to its exposure are pronounced when the body at sites like the eye, nose, skin, and throat has direct contact with the compound [1,2]. Researchers have deduced the relationship between the health effects and range of exposures, with some individuals becoming symptomatic at low levels of exposure. A few people may have gentle uneasiness while others have moderate or no inconvenience at comparative exposures. Mean level exposures are at their most elevated in the clinical dissection room or morgue [3]. Formaldehyde (FA) is a colorless, combustible and extremely reactive chemical at standard pressure and temperature [4]. It is broken down in the air and highly stable in liquid [5]. It rapidly diffuses in any tissues, e.g., the liver, through the oral or intraperitoneal route since it collaborates with various cell components [6]. Formalin was first used as a fixative and treating liquid; however, these days it is used in every field of daily life. The most appalling use of formalin is as food additive [7] and that's why there is a drastic increase in human exposure to formalin intoxication. After intake, FA is readily absorbed from the gastrointestinal tract. In the liver, FA is largely metabolized to methanol and formate by aldehyde dehydrogenase 1 or mitochondrial aldehyde dehydrogenase 2, respectively. However in high concentrations of FA, toxicity arises in the hepatocytes [8].

Research conducted on FA exposure to animals shows hepatotoxicity and abnormal histological alterations in the gastrointestinal tract [9]. A low dose of FA has been shown to be mutagenic and carcinogenic and can manifest in a wide range of toxicities in different organs [10]. Gastrointestinal cancer can also be caused by drinking water containing a high concentration of FA [11]. In growing countries like India and Nigeria, the haphazard use of FA in lots of food items and drinking water has exposed a large percentage of citizens to a huge health hazards such as liver damage [12]. The liver is a large, composite organ that performs very important tasks in sugar, fat and protein digestion. It functions in the detoxification of metabolic wastes like ammonia. Together with the spleen, it is associated with the obliteration of the remnants of the erythrocyte and the re-use of its constituents. Bile synthesis and secretion are also present in the liver, lipoproteins and plasma proteins synthesis, as well as coagulating factors. It maintains a steady level of blood glucose via glycogenesis, glycogenolysis and gluconeogenesis. The liver also plays a significant role in the elimination and detoxification of drugs. Therefore, xenobiotics (for example, liquor and numerous drugs), malnutrition, infection, and anemia, can induce liver damage [13]. Hepatic damage is a common disease that mostly occurs as a result of oxidative stress and involves progressive growth from steatosis to hepatocellular carcinoma [14].

Over 2 millennia, most Chinese medicines have made use of fungi for the management of a range of diseases [15]. Traditional oriental therapies have also benefited greatly from medicinal mushrooms, and fungal metabolites are widely used in the treatment of diseases. [16]. Additionally, mushrooms should not only be considered as food, as research has shown that they contain a lot of biologically active compounds [17]. Mushrooms have numerous compounds with some biological significance. The extensive list incorporates polysaccharides, phenolics, proteins, polysaccharide–protein complexes, lipid components, and terpenoids, alkaloids, little peptides and amino acids, nucleotides and nucleosides [18]. This extensive list refers to an extraordinary combination of organic properties, including cancer prevention agents [19], antitumor [20], antimicrobial [17], immunomodulatory [21], anti-inflammatory [22], antiatherogenic [23] and hypoglycemic activities [24]. *Gandoerma lucidum* (Lingzhi, Reishi), which has been used for quite a long time in Asian nations to improve wellbeing and advance life span, is widely perceived as a means of avoiding and treating many diseases, including malignant growth [25]. As far back as 1986, the lethal dose (LD_{50}) has been reported to be 5000 mg/kg [26]. In 2006, reports investigated the beneficial roles of *G. lucidum*. Although the vast majority of persuasive information depends on laboratory and preclinical investigations, *G. lucidum* has gained consideration in non-Asian nations [27].

Investigations with refined *G. lucidum* triterpenes have indicated in vivo results, which can be used for drug development. However, the full use of *G. lucidum* preparation in corresponding and alternative medications is progressively beneficial because the specific component of *G. lucidum* could have synergistic or added substance impacts and could influence molecular signaling pathways and targets, finally prompting the destruction of malignant cells. This study is therefore performed to examine the protective potential of *G. lucidum* in formaldehyde-induced liver damage in experimental rats.

2. Materials and Methods

2.1. Fungi Material and Extraction

The whole basidiomata of *G. lucidum* was obtained from a village in Edo state. Identification was conducted at the Department of Agricultural Sciences, Joseph Ayo Babalola University. *G. lucidum* was air dried away from the direct sun rays and samples were milled to a total of 413 g of boorish powder. In total, 200 g of the quantity of coarse powder was soaked in 1 L ethanol for 72 h, decanted and concentrated, thus yielding a dark brown extract. The extract was weighed and stored in the refrigerator.

2.2. Chemicals

Thiobarbituric acid, 1-chloro-2,4-dinitrobenzene (CDNB), 5′,5′-dithiobis-2-nitrobenzoic acid (DTNB), xynelol orange, reduced glutathione (GSH), epinephrine and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents were obtained from Cloud-Clone Inc., Wuhan, China.

2.3. Animals Care

Forty sexually matured Wistar strain male rats with the weight range of 150–200 g, were acquired in the animal colony, University of Ibadan, Nigeria. They were housed in a polycarbonate cage in an Assessment and Accreditation of Laboratory Animal care-certified animal facility and adherence to the protocol of the National Institute of Health on the Guide for the Care and Use of Laboratory Animals. Prior to acclimatization for 2 weeks, rats were kept under 12:12-h light:dark cycle and provided with NIH-07 diet and water *ad libitum*.

2.4. Experimental Design

Forty sexually matured Wistar strain male rats were divided into four groups of ten rats each and treated for thirty days (2 weeks of acclimatization inclusive) as described thus:

Group 1: were orally treated with 1 mg/mL distilled water.

Group 2: were exposed to 40% FA vapor environment for 30 min daily (the exposure was done by soaking 50 mL of FA in cotton wool and placed in a corner within the animal cage, thus exposing the animal to the vapor for a period of 2 weeks (40% FA at room temperature) [28].

Group 3: were orally treated with 100 mg/kg ethanol extract of *G. lucidum*.

Group 4: were co-administered FA and 100 mg/kg ethanol extract of *G. lucidum* (1/50 of LD_{50}). The route of administration of G. lucidum was oral and that of FA was the same as in Group 2.

Rats were then sacrificed 24 h after the last administration via cervical dislocation. Liver samples were excised, weighed, homogenized, and then processed for further experiments.

2.5. Determination of Liver Function Parameters

Blood samples were collected after sacrifice and plasma samples were obtained using the standard method. Liver function biomarkers, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglycerides, total bilirubin, direct bilirubin, albumin and cholesterol were assayed according to the manufacturer's procedure (Randox Laboratories, Crumlin, UK).

2.6. Estimation of Antioxidant and Oxidative Stress Markers

The excised liver samples were homogenized accordingly using a 50 mM Tris–KCl buffer at pH 7.4 consisting of 1.15% KCl, then further centrifuged at 12,000× *g* for 15 min at 4 °C and afterward used for biochemical assays. Estimation of the protein concentration was done according to Bradford [29]. Claiborne [30] and Misra and Fridovich [31] methods were used to determine the activities of Catalase (CAT) and superoxide dismutase (SOD). Glutathione-S-transferase (GST) activity and the GSH level were determined according to Habig et al. [32] and Rotruck et al. [33]. Meanwhile, the level of lipid peroxidation (LPO) was determined according to Jollow et al. [34]. H_2O_2 generation was determined according to the standard method of Wolff [35]. All biochemical experiments were analyzed using a SpectraMax plate reader (Molecular Device, San Jose, CA, USA).

2.7. Assessment of Inflammatory Biomarkers

Myeloperoxidase (MPO) activity was determined according to the method described by Granell et al. [36], whereas the nitrite level concentration was assessed using an established protocol [37].

2.8. Determination of Proinflammatory Cytokines

Tumor Necrosis Factor (TNF-α), IL-1β and IL-6 concentrations in liver homogenates samples were assayed using rat TNF-α, IL-1β and IL-6 Elisa kits, respectively (Cloud-Clone Inc., Wuhan, China). A microplate antibody-coated plate was provided with the kit. All reagents, samples and working standards were prepared using standard procedures as provided by the kit manufacturers.

2.9. Histological Examination

Liver samples of rats that were removed were fixed with Bouin's solution which was subsequently dehydrated in graded concentrations of alcohol. This was further cleared three times using xylene solution and was later embedded in paraffin wax. Microtome was then used to cut 4–5 mm of the paraffin waxed tissue on a slide and it was stained with haematoxylin (H) and eosin (E). The slides were then further viewed using a light microscope (Olympus CH; Olympus, Tokyo, Japan) and were snapped by pathologists.

2.10. Ethical Approval

All procedures involving animals performed in the study were performed in accordance with the ethical standards of our institution.

2.11. Statistical Analyses

Data were evaluated as mean ± SEM. Levels of statistical significance were analyzed with a one-way analysis of variance (ANOVA) which was further subjected to Bonferroni's post hoc test using GraphPad Prism 6 software. $p < 0.05$ was considered significant.

3. Results

3.1. G. lucidum Suppressed FA-Induced Reduction in Body and Organ Weight

Table 1 represents the body weight gain and relative organ weight of control, *G. lucidum* and FA-treated rats. The result shows a significant reduction in the body and organ weight of the liver of rats administered formaldehyde as compared to the control. Additionally, there was a statistically significant increase in the body and organ weight gain of rats exposed to FA and *G. lucidum* when compared to the control.

Table 1. Effect of *Ganoderma lucidum* and formaldehyde on average body weight and relative organ weight in rat.

	Control (g)	Formaldehyde (g)	*Ganoderma lucidum*	*Ganoderma lucidum* + Formaldehyde (g)
Average Body weight	42.35 ± 4.52	18.75 ± 5.45 [a]	36.74 ± 6.32	25.45 ± 4.46
Relative organ weight	7.32 ± 0.47	4.76 ± 0.78 [a]	8.76 ± 0.95	6.75 ± 0.52 [a]

a—significantly different from control.

3.2. G. lucidum Inhibits FA-Induced Alteration in Hepatic Function Enzymes

The activities of AST, ALT and ALP were significantly ($p < 0.05$) increased in the liver of rats administered FA as compared to the control. However, *G. lucidum* significantly reduced these levels when compared to the FA alone and control groups (Figure 1A–C). Additionally, the concentration of total cholesterol, direct bilirubin and total direct bilirubin was significantly increased in the liver of rats administered FA as compared to the control (Figure 1G–I). However, *G. lucidum* significantly reduced these levels when compared to the FA alone and control groups. The concentration of ALB was significantly ($p < 0.05$) decreased when compared to the control, but *G. lucidum* reversed this effect. The liver index shows an increase in rats administered FA as compared to the control. However, *G. lucidum* reversed this effect with an increase in the rats co-administered *G. lucidum* and FA (Figure 1F).

3.3. G. lucidum Attenuate FA-Induced Oxidative Damage in Rat Liver

The antioxidant enzymes activities, SOD, CAT, GST and GSH level (Figure 2A–D), and also oxidative stress indices, H_2O_2 and MDA, were tested as presented in Figure 2G–H. There was a significant decrease in the liver activities of SOD, CAT, GST and the level of GSH in rats administered FA alone as compared to the control rats. Conversely, the co-administration of *G. lucidum* restored the level and activities of these enzymes. Furthermore, there was a significant increase in the levels of oxidative stress markers (H_2O_2 and MDA) in the liver of rats administered FA. However, the rats co-administered with *G. lucidum* revealed a significant decrease in the levels of H_2O_2 and MDA in the testes of treated rats as compared to FA alone as shown in Figure 2A,B.

3.4. G. lucidum Ameliorate FA-Induced Inflammation in Rat Liver

Nitrite level and the activity of MPO were determined in the liver of rats as shown in Figure 2E,F. An administration of FA alone resulted in a significant elevation of nitrite level and activity of MPO as compared to the control rats. On the contrary, rats co-administered with *G. lucidum* had a significantly decreased nitrite level and MPO activity compared to the livers of FA-administered rats. However, the administration of *G. lucidum* alone did not have an effect on nitrite level and the activity of MPO. Additionally, proinflammatory cytokines (TNF-α, MPO and IL-1β) were significantly

($p < 0.05$) increased in FA-administered rats when compared to the control as shown in Figure 2I–K; however, *G. lucidum* reversed this effect with a significant ($p < 0.05$) decrease in the proinflammatory cytokines as compared to the control.

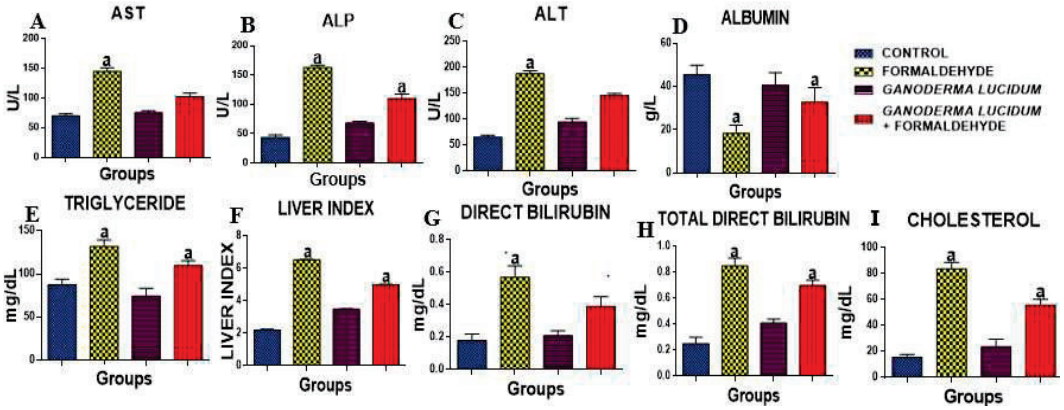

Figure 1. Effect of formaldehyde and *Ganoderma lucidum* on hepatic enzyme markers. Data are presented as mean ± SD, $n = 7$; a: $p < 0.05$ vs. control; (**A**) AST (**B**) ALP (**C**) ALT (**D**) ALB (**E**) TRIG (**F**) LIVER INDEX (**G**) DIRECT BIL (**H**) TOTAL BIL (**I**) CHOL. AST: Aspartate aminotransferase, ALP: Alkaline Phosphatase, ALT: Alanine aminotransferase.

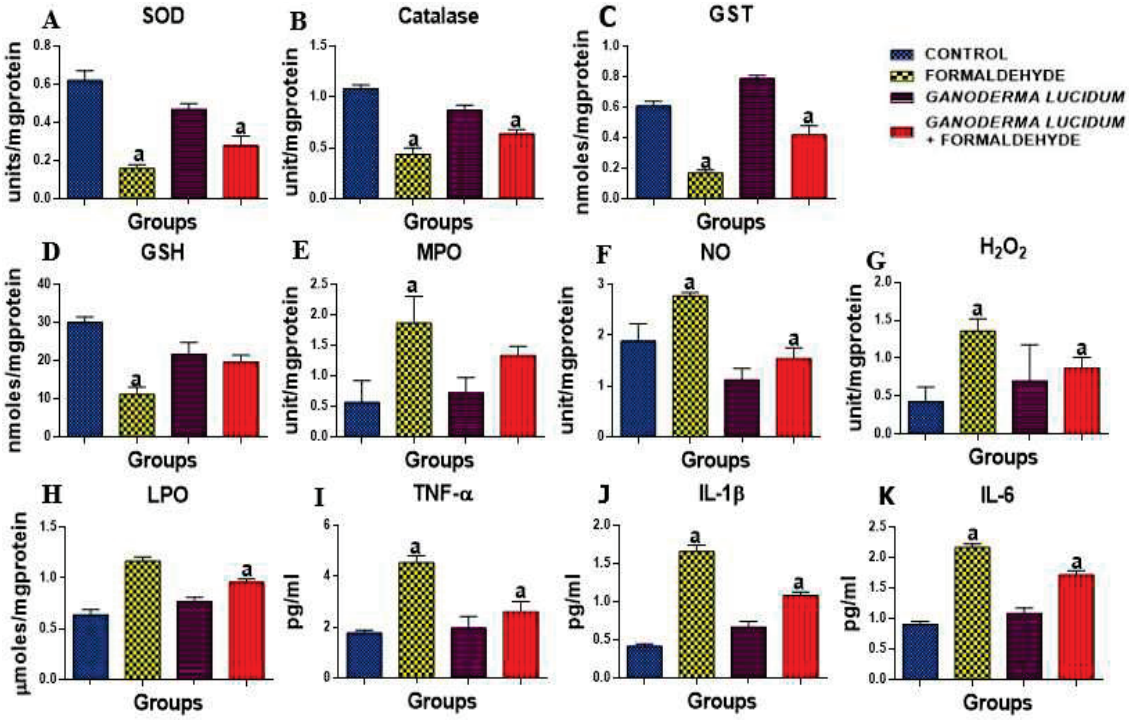

Figure 2. Effect of formaldehyde and *Ganoderma lucidum* on oxido-inflammatory markers. Data are presented as mean ± SD, $n = 7$; a: $p < 0.05$ vs. control, (**A**) SOD: superoxide dismutase, (**B**) CAT: catalase (**C**) GST: Glutathione-s-transferase (**D**) GSH: reduced glutathione, (**E**) MPO: myeloperoxidase, (**F**) NO: Nitric oxide (**G**) H_2O_2: Hydrogen peroxide (**H**) LPO: lipid peroxidation (**I**) TNFα: Tumor necrosis factor α. (**J**) IL-1β: Interleukin 1β, (**K**) IL-6: Interleukin 6.

3.5. Histopathological Observations

Figure 3 shows the histological structure of the representative photomicrograph of the liver. The control rats and the *G. lucidum*-treated rats show a normal architecture. However, rats treated with FA alone show a diffuse periportal cellular infiltration with severe congestion indicating hepatic damage. Additionally, histological structures of the liver of rats co-administered with formaldehyde and *G. lucidum* at 100 mg/kg showed relatively normal features.

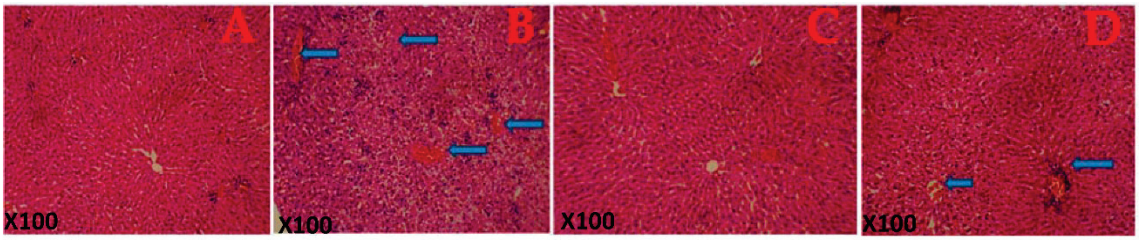

Figure 3. Histological architecture of the liver of the rat in each group. (**A**) Control rat shows the normal architecture of the liver. (**B**) Rats administered Formaldehyde (FA) alone shows a diffuse periportal cellular infiltration with severe congestion indicating hepatic damage. (**C**) Rats administered *Ganoderma lucidum* show a normal architecture of the liver while (**D**) rats co-administered with formaldehyde and *Ganoderma lucidum* shows mild congestion. Magnification: X100.

4. Discussion

The use of natural products in the prevention and management of various illnesses has prominently increased in the last few years [38]. The present study established the promising chemopreventive potential of *G. lucidum* in the liver, preventing liver damage caused by FA exposure. The reestablishment of unhealthy liver functions was evident by the remarkable loss of body weight, and a significant reduction in the liver organ weight which can result from shrinkage in the liver as seen in the FA-administered group (Table 1), but this was prevented in the rats treated with *G. lucidum*. The hepatoprotective potential of *G. lucidum* against FA was investigated by determining ALT, AST and ALP. ALT is the important liver damage enzyme that catalyzes transamination reactions. The occurrence of conditions that can cause liver damage such as cancer, injury and hepatitis, will result in higher levels of this enzyme [14]. AST and ALP, the biomarkers of liver damage, are cytosolic and mitochondrial enzymes whose levels are usually increased in cases of chronic illness and necrosis due to loss of hepatocellular integrity. These enzymes are involved in the transfer of α-amino groups from alanine and aspartate to the α-keto group of ketoglutarate to form pyruvate and oxaloacetate, respectively [39]. As shown in figures, there is a significant increase ($p < 0.05$) in the levels of these enzymes in the group administered FA when compared to the control. However, treatment with 100 mg/kg *G. lucidum* significantly reduced the elevated levels, showing that *G. lucidum* exhibits a protective role against FA-induced liver damage in rats. This study is related to that of Lakshmi et al. [40] that showed the effects of *Ganoderma lucidium* on hepatic damage induced by benzo(a) pyrene. The elevated liver function enzymes were significantly reduced by *Gandoerma lucidum* administration.

Albumin is a measure of the synthetic function of the liver. A significant decrease in the albumin level in the FA-administered group could be traced to the reduction in protein synthesis that is an effect of FA. The carbonyl atom of FA reacts with the amino groups (nucleophilic sites) on the cell membranes forming hydroxymethyl amino acid derivatives [41]. However, treatment with 100 mg/kg *G. Lucidum* significantly increases the level of albumin. Cholesterol oxidation causes enzymatic increases in bile acids and contributes to hepatic cholesterol accumulation and hepatocellular injury. This is further explained by its significant increase in the FA-administered group as compared to the control. However, *G. Lucidum* treatment reduced the elevated cholesterol levels significantly when compared to the control. Total direct bilirubin is also an indicator of the destruction of erythrocytes and the proper functioning of the liver, gallbladder and bile ducts, and is a potential marker for liver damage. Triglycerides were also increased in

the FA-administered group as compared to the control. However, *G. Lucidum* reduced the elevated cholesterol levels significantly when compared to the control. The liver index, the indicator of hepatic manifestation of metabolic disorders, was upregulated in the group administered FA, thus showing an impairment of the liver. However, upon administration of 100 mg/kg *G. lucidum* to the group induced with 40% FA, there was a significant downregulation in the increased liver index.

When the body metabolism is impaired, an increase in the production of toxic molecules such as free radicals and antioxidants, known as free radical scavengers, are needed to reduce or neutralize the free radical formation [42]. Our results show that FA has a direct effect on the hepatocytes and also an indirect effect through the circulatory and immune systems [43]. The hepatic destruction caused by FA causes oxidative stress and produces reactive oxygen species (ROS), as shown in the significant increase in H_2O_2 and LPO, which are known to be oxidative stress markers, and also a decrease in GSH, GST, catalase and SOD, which are antioxidant markers. These observations are accordance to Payani et al. [44] who reported that FA exposure significantly reduced the levels of enzymatic and non-enzymatic antioxidants. However, *G. lucidum* significantly increases the activities and levels of these antioxidant markers. These results indicated that animals treated with *G. Lucidum* cause a significant increase in the levels of antioxidant enzymes. These results are in line with the reports of other researchers that allude to the fact that *Gandoderma lucidium* has antioxidant activities both in vivo and in vitro [45–47]. These results indicated the hepatoprotective efficacy of *G. Lucidum.* Myeloperoxidase is one of the most important molecules released after the recruitment and activation of phagocytes and it is involved in the production of oxidative stress. Additionally, proinflammatory cytokines activate iNOS during liver injury to abnormally producing NO that contributes immensely to the pathogenesis and evolution of liver damage. The present study shows a distinct increase in the activity and level of MPO and NO in FA-administered rats' livers as compared to the control. However, the reduced level of MPO and NO following *G. lucidum* treatment shows its potential to prevent inflammation in the liver of rats [48,49].

TNF, IL-1β and IL-6 play a major role in the pathogenesis of liver damage. TNFs are majorly a group of proinflammatory cytokines known to perform a crucial role in the instigation of liver damage with evidence that oxidative stress might act in conjunction with endotoxins to augment TNF production [50]. Interleukin 1β and 6 are potential biomarkers of acute or chronic liver toxicity. TNF, IL-1β and IL-6 are proinflammatory cytokines that are released into the bloodstream from the liver during hepatic toxic injury. Thus, biological agents suppressing these cytokines are known to have demonstrated huge therapeutic potential. As shown in our results, there was a significant upregulation in the levels of these cytokines in rat livers when administered FA. The significant downregulation of the levels of the cytokines was demonstrated in the group treated with 100 mg/kg *G. Lucidum*. This further indicates the hepatoprotective efficacy of *G. Lucidum.* The above result corroborates with the histopathological finding as shown in Figure 3 as rats administered FA show a diffuse periportal cellular infiltration with severe congestion indicating hepatic damage; however, *G. lucidum* was able to reverse this effect [51].

5. Conclusions

The results from this study demonstrated that exposure to FA led to a significant decline in antioxidant markers [52], with a concomitant increase in liver transaminases, lipid profile as well as inflammatory markers [53]. Additionally, *G. lucidum* possesses protective roles as it has the ability to restore antioxidant, lipid profile and anti-inflammatory statuses. Hence, *G. lucidum* may be a probable drug candidate to target liver damage [54,55].

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Piperine Regulates Nrf-2/Keap-1 Signalling and Exhibits Anticancer Effect in Experimental Colon Carcinogenesis in Wistar Rats

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Abstract: Colon cancer is the most common cancer in men and women globally, killing millions of people annually. Though there widespread development has been made in the management of colorectal cancer, still there is an urgent need to find novel targets for its effective treatment. Piperine is an alkaloid found in black pepper having anticancer, anti-inflammatory activities, safe and nutritive for human consumption. Nuclear factor-erythroid 2–kelch-like ECH-associated protein 1(Nrf-2/Keap-1)/Heme-oxygenase1 (HO-1) signaling pathway plays a vital part in shielding cells from intracellular oxidative stress and inflammation. A potential cross-talk between the Nrf-2 and NF-κB pathways is recognized during cancerous growth and expansion. We studied this pathway extensively in the present study to discover novel targets in the prevention of chemically induced colon cancer with piperine to simulate human colon cancer pathology. Animals were divided into four groups. Groups1 and 2 were used as a negative control and positive control where 1,2–Dimethylhydrazine, DMH was administered in group 2, while group 3 and 4 were prevention groups where piperine at two different doses was given two weeks prior to DMH and continued until end of experiment. We found that piperine inhibited NF-κB by the activation of Nrf-2, blocking downstream inflammatory mediators/cytokines (TNF-α, IL-6, IL-1β, Cox-2, PGE-2, iNOS, NO, MPO), triggering an antioxidant response machinery (HO-1, NQO-1, GSH, GR, GPx, CAT, SOD), scavenging ROS, and decreasing lipid peroxidation. Histological findings further validated our molecular findings. It also downregulates CEA, MDF and ACF, markers of precancerous lesions in colon, alleviates infiltration of mast cells and depletes the mucous layer. Our results indicate that piperine may be an effective molecule for the prophylactic treatment of colon carcinogenesis by targeting the NF-κB/Nrf-2/Keap-1/HO-1 pathway as a progressive strategy in the preclusion and effective treatment of colorectal cancer.

Keywords: piperine; colon carcinogenesis; NF-κB/Nrf-2/Keap-1/HO-1 signalling pathways; ACF; MDF

1. Introduction

According to Globocon 2018 data from Saudi Arabia, Colorectal cancer (CRC) is documented as the top most common cancer in men, third most common cancer-caused death in women, and fourth most frequently occurring cancer throughout the world (Globocon 2018). Surgery, radiotherapy, and chemotherapy are the conventional strategies of treatment for CRC, which fail because patients either develop drug resistance or excessive adverse effects which become fatal in themselves [1,2]. Hence, there is an imperative to mitigate adverse effects of the present treatment regimen. In this sense, environmental factors, explicitly dietary trends, contribute significantly towards the aetiology of colon cancer incidences in various populations. Drug discovery paradigms have shown remarkable results by using natural products. Therefore, natural compounds possess various organically potential molecules which have been used to either converse, inhibit, or avert early cancer stages or the promotion of precancerous cells like inflammation, transformation, and proliferation to metastatic disease. Nevertheless, many natural product-based drugs have been isolated from nature due to their lower toxicity, easy availability, affordability and targeted nature. A number of drugs have been isolated from natural sources owing to their great chemical diversity, which may be a consequence of millions of years of evolutionary process to combat biotic and abiotic stresses. In the past, plants have been used as an enriched source of drugs and will further deliver state-of-the-art chemical frameworks for mitigation of diseases [3].

Epidemiological reports demonstrate that CRC cases are commonly sporadic, which may be associated with nutrition and diet-influencing oxidation reactions in cells and tissues, by activating a cascade of molecular events in them. Oxidative stress deregulates normal functioning of cells by inducing DNA damage, mutations, peroxidation of lipids and proteins. Moreover, a close association between biomarkers of oxidative stress, nitrosative stress and progression of CRC has been unraveled by clinical studies [4–7]. Inflammation is a normal biological response of our body at the time of infection or damage. It stimulates the immune system to release chemical molecules called pro-inflammatory signals which protect our body. Nevertheless, too much inflammation is a bad representation as well. There is an association between carcinogenesis and inflammation as inflammation is one of the hallmarks of cancer. Cancer progression is exacerbated by inflammatory processes via averting the differentiation of cells and promoting tumor formation and expansion [8]. In the present study, we used DMH to induce colon carcinogenesis where DMH is metabolized in the liver to azoxymethane and, after subsequent metabolization, forms methylazoxymethanol (MAM). Glucuronic acid conjugates with MAM, forming glucuronide MAM which, with the help of blood or bile, is released into the colon. Following deconjugation by intestinal microbial enzymes like β-glucuronidase, electrophilic methyldiazonium ion is produced as a by-product of MAM metabolization. Thereafter, the methyldiazonium ion produces carbonium ion which methylates DNA and other biomolecules in the epithelial layer of colon tissue, leading to procarcinogenic events resulting from inflammation and tumor promotion [9].

Piperine is an interesting compound present in the fruits and roots of *Piper nigrum* L. (black pepper) and *Piper longum* (long pepper) containing 3–9% content of black pepper. It is the most prevalent dietary amide alkaloid, having anti-inflammatory, immunosuppressive, anti-cancer, neuroprotective, and anti-oxidant potential. Lately, piperine was found to suppress the promotion of human colon cancer cells, prostate cancer cells, cytotoxic for human and murine melanoma cells [10]. It was also found to enhance the efficacy of anti-cancer drugs by alleviating drug resistance remarkably [11]. In traditional Indian medicine, black pepper is used extensively and is beneficial because of the piperine in it [12,13]. The bioavailability of the drug, inhibition of the drug transporter and cytochrome P450 results in an increase in absorption caused by Piperine. Piperine was found to decrease protein damage

and impede cell proliferation in benzo(a)pyrene-induced lung cancer in an animal model. It also repressed stem cell renewal in breast tissue. It was found to be cytotoxic to human rectal cancer cells (HRT-18), androgen-dependent prostate cancer cells (LNcaP), androgen-independent prostate cancer cells (PC-3), HER2-overexpressing breast cancer cells (SKBR3), mouse mammary carcinoma cells (4T1), and mouse melanoma cells (B16F-10) [14]. Moreover, it is reported to suppress angiogenesis and regulates multidrug resistance in breast and lung cancer cells to respond to traditional anti-cancer drugs. The current study was done to explore the role of piperine, keeping in mind the cytotoxic, anti-proliferative and anti-cancer potential, using Dimethyl hydrazine (DMH)-induced colon cancer in Wistar rats [10,14,15], thereby highlighting the efficacy of piperine in various cancer cells including colon cancer. However, the exact mechanism of anti-carcinogenic action of piperine is unknown and more research needs to be done to elucidate it. As per our knowledge, our study is the first to decipher the role of piperine in mitigating experimental colon cancer in Wistar rats by regulating cross-talk between the Nuclear factor kappa light chain enhancer of activated B cells (NF-kB-p65) and Nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway to discover novel targets for the prevention of chemically induced-colon cancer which simulates human colon cancer pathology. We extensively studied inflammatory mediators and markers of inflammation like NF-κB, Tumor Necrosis Factor Alpha (TNF-α), Interleukin 6 (IL-6), cyclooxygenase-2 (Cox-2), prostaglandinE-2 (PGE-2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), myeloperoxidase (MPO); markers of oxidative stress like reactive oxygen species, lipid peroxidation; depletion of anti-oxidant machinery, activation of Nrf-2 pathway and its downstream molecules, which trigger antioxidant response machinery like Heme oxygenase-1 (HO-1), NADP(H) quinone oxidoreductase-1 (NQO-1), reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), which has never been done before using piperine in a colon cancer model. We are the first to unravel the downregulation of markers of precancerous lesions in the colon like carcinoembryonic antigen (CEA), mucin-depleted foci (MDF), aberrant crypt foci (ACF), mast cells expansion and the depletion of mucous layer with prophylactic treatment of piperine. Therefore, the current work was planned to assess the beneficial properties of piperine on colon cancer in vivo to further illuminate its molecular mechanism involving the Nrf-2/Keap-1/NF-κB pathway.

2. Materials and Methods

2.1. Chemicals

Piperine, 1,2 dimethyl hydrazine (DMH) and other chemicals were obtained from Sigma Aldrich and were of greatest purity grade.

2.2. Animals

For the animal study, 4–6-week-old male Wistar rats, weighing 120–150 g were obtained from Institutional Animal Housing Facility. Rats were kept under typical laboratory environment with 45–55% relative humidity, 23–25 °C temperature and 12 h light/12 h dark period, having free access to standard diet and tap water during the experimental tenure. All procedures for using experimental animals were checked and proper permission was obtained from the "Institutional Animal Ethical Committee (IAEC)" (Approval No, Au/FVS/PS-57/9713) which is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), New Delhi, India.

2.3. Preparation of Carcinogen

A total of 1 mM EDTA was used as a vehicle to dissolve DMH in distilled water. The pH was adjusted to 6.5 with 1 M NaOH solution to check the steadiness of the chemical before administering it to rats.

2.4. Experimental Regimen

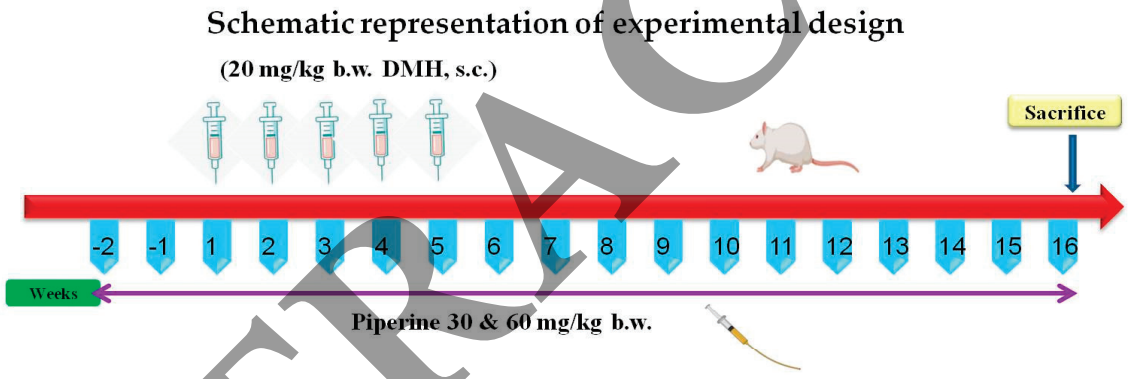
Group I: Rats had access to standard diet and drinking water along with physiological saline given orally.

Group II: In the current group, rats had access to standard diet and drinking water. Rats were given 20 mg/kg b.wt. (b.wt.) DMH injection given subcutaneously in the groin once weekly for 5 weeks.

Group III: Piperine and DMH treated group. In this group, rats were prophylactically treated with Piperine (30 mg/kg b.wt.) two weeks prior to the start of DMH injection, which then continued five times a week along with 20 mg/kg b.wt. subcutaneous DMH injection as given in group II. Piperine treatment was continued till the end of 16th week.

Group IV: Piperine and DMH treated group). In this group, rats were prophylactically treated with Piperine (60 mg/kg b.wt.) two weeks prior to the start of DMH injection, which then continued five times a week along with 20 mg/kg b.wt. subcutaneous DMH injection as given in group II. Piperine treatment was continued till the end of 16th week.

All the animals were sacrificed at the end of the 16th week (Figure 1). The chemo-preventive potential of Piperine was assessed for mitigating colon tissue injury and the initiation of precancerous lesions/events/biomarkers of colon carcinogenesis via checking ROS measurement, anti-oxidant armory, carcinoembryonic antigen (CEA), mucin-depleted foci (MDF), aberrant crypt foci (ACF), NF-κB /Nrf-2/Keap-1/HO-1 pathway proteins were measured, and histological alterations were studied.



Group I: physiological saline orally
Group II: 20 mg/kg b.w. DMH injection (s.c.) in the groin once weekly for 5 weeks
Group III: Prophylactic treatment of Piperine orally (30 mg/kg b.w.) five times a week, two weeks prior to the start of DMH injection until 16th week +20 mg/kg b.w. DMH injection (s.c.) in the groin once weekly for 5 weeks
Group IV: Prophylactic treatment of Piperine orally (60 mg/kg b.w.) five times a week, two weeks prior to the start of DMH injection until 16th week +20 mg/kg b.w. DMH (s.c.) in the groin once weekly for 5 weeks
All the animals were sacrificed at the end of 16th week
s.c: subcutaneously; b.w: body weight

Figure 1. Represents treatment schedule of the study.

2.5. Post–Mitochondrial Supernatant (PMS) Preparation

Colons were cleaned immediately and perfusion was done by cold saline. Colons were homogenized in a homogenizer in chilled phosphate buffer (0.1 M, pH 7.4) (10% *w*/*v*). To separate the nuclear debris, centrifugation was done at 700× *g* in cooling centrifuge to homogenized colons for 10 min. PMS in the form of aliquot, thus obtained, was used as a repertoire of numerous enzymes [16].

2.6. Estimation of Carcinoembryonic Antigen (CEA)

CEA was measured by commercially available kit from USCN Life Science Inc. CEA enzyme levels in serum was measured by commercially available kit ARCHITECT CEA enzyme chemiluminscent microparticle immunoassay test kit (Abbott, Ireland Diagnostic Division, Sligo, Ireland) as per the manufacturer's protocol.

2.7. Estimation of Aberrant Crypt Foci (ACF)

ACF were measured by the method [15]. Randomly, colons were selected and stained for 6 min in a 0.05% filtered solution of methylene blue. ACFs were counted under light microscope at 40× magnification per colon.

2.8. Estimation of Mucin-Depleted Foci (MDF)

MDF were measured by the method [15]. After measuring ACF, Colons were stained with high-iron diamine-alcian blue (HID-AB) to assess mucin production. MDF were counted per colon under light microscope at 40× magnification.

2.9. Immuno-Histochemical Staining pNF-kB-p65, Nrf-2, Keap-1, HO-1 and NQO-1

Immunohistochemical staining protocol was followed as described [17]. We used the following anti-bodies: anti-rat NF-kB-p65 rabbit antibody (dilution 1:100), anti-rat Nrf-2 rabbit antibody (dilution 1:100), anti-rat keap-1 polyclonal antibody (dilution 1:200), anti-rat HO-1 polyclonal antibody (dilution 1:200) and anti-rat NQO-1 (1:250) overnight at 4 °C. The same procedure was followed as previously described. Finally, slides were visualised under light microscope.

For semi quantitative Evaluation of pNF-kB-p65, Nrf-2, Keap-1, HO-1and NQO-1, the tissue sections were categorized as 0 (no staining), 1 (staining, 25%), 2 (staining between 25% and 50%), 3 (staining between 50% and 75%), or 4 (staining >75%), depending upon diffusivity of the DAB. As regards intensity of staining, colon tissue sections were classified as follows: 0 (nil staining) 1 (staining is detectable, but weak), 2 (staining is distinct), or 3 (staining is intense). Scores for immunohistochemical staining were determined by adding the diffusivity and scores of staining intensities. Slides were seen by two independent observers who did not know about the experimental protocol. The slides with variable evaluations were re-evaluated, and a consensus was achieved. Measurements were carried out using an Olympus BX51 microscope having objectives of magnification 10 and 40×.

2.10. Estimation of Reactive Oxygen Species (ROS)

ROS was estimated as explained [18]. Based on the oxidation of 2′7′dichlorodihydrofluorescein diacetate to 2′7′-dichloro-fluorescein, ROS levels were measured. ROS levels were measured based on the oxidation of 2′7′dichlorodihydrofluorescein diacetate to 2′7′-dichloro-fluorescein.

2.11. Estimation of MDA

Lipid peroxidation (LPO) was measured by the following method [19]. The reaction mixture contained 580 µL phosphate buffer of 0.1 M, pH 7.4, 200 µL PMS, 200 µL ascorbic acid of 100 mM, and 20 µL ferric chloride of 100 mM, forming a total volume of 1 mL. This reaction was kept in a shaking water bath for 1 h to incubate at 37 °C. By adding 10% of 1 mL, the trichloroacetic acid reaction was stopped, followed by the addition of 0.67% of 10 mL thiobarbituric acid (TBA), and all the reactions in tubes/vials were placed in a boiling water bath for a period of 20 min. The tubes/vials of the above-mentioned reaction mixture were transferred to an ice bath and then centrifuged at 2500× *g* for 10 min. The optical density of the supernatant at 535 nm was measured by the amount of malondialdehyde (MDA) formed in each of the samples. The results were expressed as nmol TBA formed/h per g tissue at 37 °C using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.12. Estimation of Antioxidant Enzyme Armory

2.12.1. Measurement of Superoxide Dismutase Activity (SOD)

SOD was evaluated by method [20]. The total reaction mixture is 3 mL with 2875 µL Tris–HCl buffer of 50 mM of pH 8.5, pyrogallol with 24 mM in 10 mM-HCl, and 100 µL PMS. Finally, enzyme activity was expressed as units/mg protein at 420 nm.

2.12.2. Measurement of Catalase Activity (CAT)

CAT was determined by method [21]. The reaction mixture contains 3 mL with 1950 µL phosphate buffer of 0.1 M with pH 7.4, 1000 µL H_2O_2 of 0.10 mM, and 50 µL of 10% PMS. Absorbance changes are measured at 240 nm and enzyme activity is calculated as nmol H_2O_2 consumed/min/mg protein.

2.12.3. Measurement of Reduced Glutathione (GSH)

GSH in colon was measured by the method [22]. A total of 10% PMS fraction of 1000 µL was combined with 1000 µL of 4% sulphosalicylic acid. The reaction mixture was incubated at 4 °C for 60 min at least and then centrifuged at 1200× *g* for 15 min at 4 °C. The reaction mixture consisted of total volume of 3 mL with 400 µL filtered aliquot, 2200 µL phosphate buffer of 0.1 M with physiological pH and 400 µL DTNB of 10 mM. The yellow color developed was read immediately at 412 nm.

2.12.4. Measurement of Glutathione Reductase (GR) Activity

GR activity was determined by method [23]. The reaction mixture contained a total volume of 2 mL with 1650 µL phosphate buffer of 0.1 M, at pH 7.6, 100 µL EDTA of 0.5 mM, 50 µL oxidized glutathione of 1.0 mM, 100 µL NADPH of 0.1 mM and 100 µL of 10% PMS in a total volume of 2.0 mL. The enzyme activity was assessed at 25 °C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/ mg protein using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

2.12.5. Measurement of Glutathione Peroxidase Activity (GPx)

GPx was calculated by method [24]. The reaction mixture contained 100 µL EDTA of 1 mM, 100 µL sodium azide of 1 mM, 1440 µL phosphate buffer of 0.1 M with pH 7.4, 50 µL glutathione reductase (1 IU/mL), 50 µL reduced glutathione of 1 mM, 100 µL NADPH of 0.2 mM and 10 µL H_2O_2 of 0.25 mM and 100 µL of 10% PMS. The consumption of NADPH at 340 nm was measured at room temperature. GP x was calculated as nmol NADPH oxidized/min/mg protein with a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

2.13. Estimation of Nitric Oxide (NO)

NO content was determined by method [25] At room temperature, various concentrations of piperine in phosphate buffer solution of pH 7.4 were incubated for 5 h with an equal volume of sodium nitroprusside solution of 5 mM in phosphate buffer of pH 7.4. Similarly, various concentrations of ascorbic acid (25–400 µg/mL) in phosphate buffer of pH 7.4 were also incubated with an equal volume of sodium nitroprusside solution (5 mM) in phosphate buffer (pH 7.4). Controlled experiments had no test compound, but instead an equal volume of buffer was used. Once incubation was done, 500 µL of incubation mixture was combined with 500 µLof Griess' reagent (sulphanilamide 1%, *o*-phosphoric acid 2%, and naphthyl ethylene diamine dihydrochloride 0.1%), and the absorbance was taken at 546 nm.

2.14. Estimation of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2 standard curve) was assessed by method [26]. A total of 2000 µL microsomes were suspended and incubated at 37 °C for 60 min in 1000 µL reaction mixture containing

phenol red of 0.28 nm, horseradish peroxidase, dextrose of 5.5 nm, and phosphate buffer of 0.05 M with pH 7.0. A total of 10 µL of NaOH with 10 N was used to stop the reaction and then centrifuged at 800× *g* for 5 min. The absorbance of the supernatant was recorded at 610 nm against a blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2 per hour per g tissue based on the standard curve of H_2O_2-oxidized phenol red.

2.15. Protein Estimation

The protein estimation was done by method [27] using BSA as standard.

2.16. Estimation of Myeloperoxidase (MPO)

The neutrophil quantification was measured as a level of MPO activity and was carried out using the Bradley et al. method [28].

2.17. Estimation of Tumor Necrosis Factor Alpha (TNF-α), Interleukin 6 (IL-6), Cyclooxygenase-2 (Cox-2), Prostaglandin E-2 (PGE-2), and Inducible Nitric Oxide Synthase (iNOS)

TNF-α, IL-6, PGE-2, iNOS and Cox-2 were measured by using commercial kit (eBioscience, Inc., San Diego, CA, USA). Assays were performed as per the instructions of the manufacturer provided with the kit on an Elisa Plate Reader.

2.18. Alcian Blue–Neutral Red (AB-NR) Staining for Mucin Analysis

This procedure was done as described [15]. Four-millimetre colon sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-L-lysine coated microscopic slides. Dewaxing of paraffinized sections in xylene and rehydration in graded series of ethanol to water was done, followed by staining with 1% Alcian blue of pH 2.5 in 3% acetic acid solution for half an hour and then rinsing for 1 min in 3% acetic acid solution to prevent nonspecific staining followed by washing in distilled water and the sections are then counterstained with neutral red (0.5% aqueous solution) for 20 s, then dehydration in alcohol and mounting using mounting media is completed, and finally slides are visualized under a light microscope.

2.19. Statistical Analysis

The data are presented as the mean ± standard error of the mean (SEM) from all the individual groups. Analysis of variance (ANOVA) has been used to show the differences between groups followed by Tukey–Kramer multiple comparisons test, and the minimum criterion for statistical significance is set at $p < 0.05$ for all comparisons.

3. Results

3.1. Piperine Treatment Mitigates CEA Production

There was an increase in CEA in tumor group rats when compared with control rats (*** $p < 0.001$). However, treatment with Piperine treatment diminished levels of CEA in group II and IV significantly ($^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$), as shown in Figure 2.

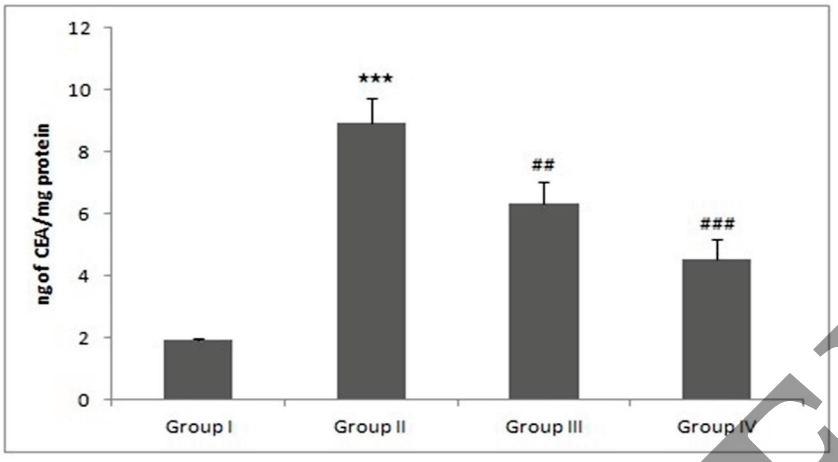


Figure 2. Piperine treatment mitigates CEA production. In group-II, the CEA level was increased significantly (*** $p < 0.001$) as compared to control group. Treatment with Piperine (30 and 60 mg/kg b. wt.) significantly attenuated CEA level in group III (## $p < 0.01$) and group IV (### $p < 0.001$) as compared to group II ($n = 10$).

3.2. Piperine Treatment Mitigates ACF and MDF, Precancerous Lesion Markers

ACF and MDF developed by DMH administration are early events which lead to colon carcinogenesis. In the current study, there was increased ACF and MDF in the tumor group when compared with control group, although treatment with piperine reduced ACF and MDF scores in group III and group IV animals significantly, as shown in Figure 3A,B.

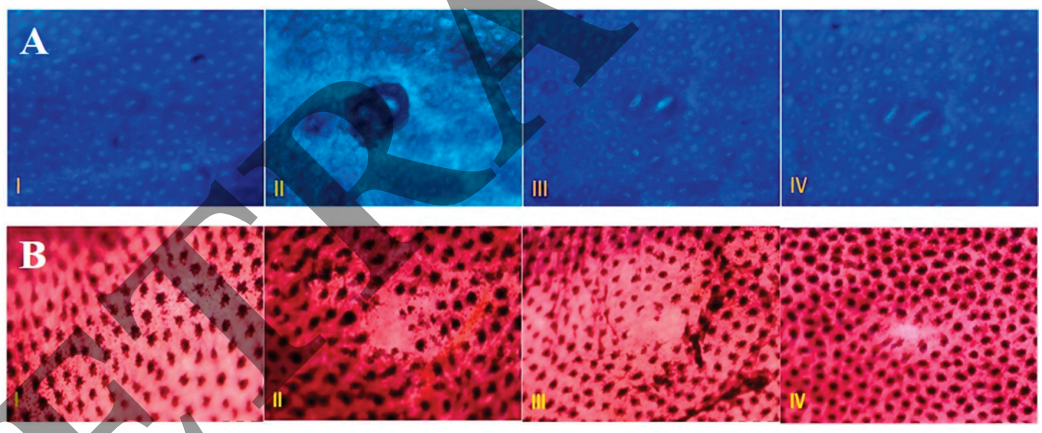


Figure 3. Effect of piperine treatment on ACF and MDF. (**A**) Piperine attenuates ACF per rat colon in DMH administered groups as compared to tumor group. ACF visualized by methylene blue (MB) staining are detectable under microscope. The colons here were opened and stained with high iron diamine (HID) and Alcian blue (AB) as we can see in the picture. (**B**) Piperine attenuates MDF per rat colon in DMH administered groups as compared to tumor group. MDF are the dysplastic crypts lacking mucin formation found in the colons of chemical carcinogen rodent studies. The colons here were opened and stained with high iron diamine (HID) and Alcian blue (AB) as we can see in the picture ($n = 10$).

3.3. Piperine Treatment Regulates Nrf-2/Keap-1/HO-1/NQO-1 Pathway

The immunohistochemical slides show strong expression of Keap-1(Figure 4B) and lower expression of Nrf-2 (Figure 4A), NQO-1 (Figure 4C) and HO-1(Figure 4D) in DMH group as compared to control. However, piperine treatment induced Nrf-2, NQO-1 and HO-1 expression in colon tissue in rats as compared to group II, where no significant staining was observed. Moreover, Piperine (60 mg/kg

b.wt.)-treated rats showed lesser staining of Keap-1 as compared to DMH-treated rats which infers the anti-inflammatory potential of Piperine and increased Nrf-2, NQO-1, and HO-1, causing anti-oxidant machinery to function and shutting down NF-κB and its downstream elements (Figure 4A–D).

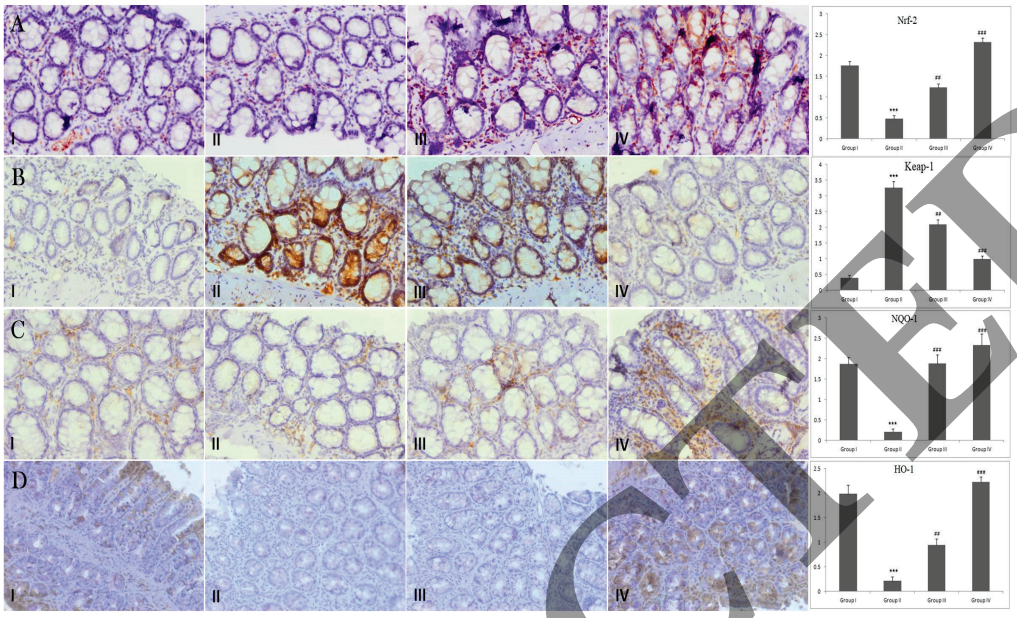


Figure 4. Effect of piperine treatment on Nrf-2, Keap-1, NQO-1 and HO-1expression. Photomicrographs of colon sections depicting immunohistochemical analyses. Adjacent to photomicrographs are four panels which show quantitative evaluation of Nrf-2, Keap-1, NQO-1 and HO-1 expression immunostaining. Significant differences were indicated by *** $p < 0.001$ when compared with group I and ($^{\#\#}$ $p < 0.01$), ($^{\#\#\#}$ $p < 0.001$) when compared with group II (**A**) Brown color indicates specific immunostaining of Nrf-2, and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has decreased immunopositive staining of Nrf-2, as indicated by brown color, as compared to control group-I, while treatment of Piperine (30 and 60 mg/kg b. wt.) in groups-III and IV increased Nrf-2 compared to group II. Piperine significantly activated Nrf-2 in group III and IV, respectively, ($^{\#\#}$ $p < 0.01$) and ($^{\#\#\#}$ $p < 0.001$), when compared with DMH-administered group-II. (**B**) Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of Keap-1 and light blue color indicates counter staining by hematoxylin. The colonic section of DMH-administered group-II has more Keap-1, as indicated by brown color, as compared to control group I, while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV reduced Keap-1 immunopositive as compared to group II. Piperine significantly suppressed Keap-1 in group III and IV, respectively, ($^{\#\#}$ $p < 0.01$) and ($^{\#\#\#}$ $p < 0.001$), when compared with DMH-administered group-II. (**C**) Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of NQO-1 and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has decreased immunopositive staining of NQO-1, as indicated by brown color, as compared to control group I, while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV increased NQO-1 as compared to group II. Piperine significantly upregulated NQO-1 in group III and IV, respectively, ($^{\#\#\#}$ $p < 0.001$) when compared with DMH-administered group-II. (**D**) Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of HO-1 and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has decreased immunopositive staining of HO-1, as indicated by brown color, as compared to control group I while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV increased HO-1 as compared to group II. Piperine significantly upregulated HO-1 in group III and IV, respectively, ($^{\#\#}$ $p < 0.01$) and ($^{\#\#\#}$ $p < 0.001$), when compared with DMH-administered group-II. All images have original magnification of 40× ($n = 10$).

3.4. Piperine Treatment Mitigates ROS

ROS was pointedly higher (*** $p < 0.001$) in group II (DMH group) when compared with group I, suggesting that DMH increases oxidative stress by initiating ROS. Piperine treatment reduced ROS in colon tissue in group III (## $p < 0.01$) and group IV animals, respectively (### $p < 0.001$) (Figure 5).

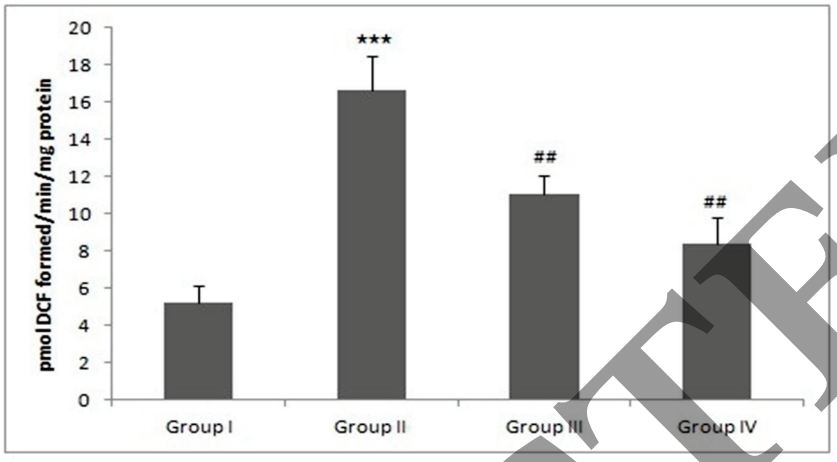


Figure 5. Piperine treatment mitigates ROS. In DMH-administered group-II, a tremendous amount of ROS was produced (*** $p < 0.001$) as compared to group-I. Treatment with piperine (30 and 60 mg/kg b. wt.) significantly mitigated ROS levels in group III (## $p < 0.01$) and group IV (## $p < 0.01$) as compared to group II ($n = 10$).

3.5. Piperine Treatment Alleviates MDA Levels

MDA is a by-product and hallmark of lipid peroxidation. There was a sharp rise in MDA in the DMH-administered/tumor group as compared to control (*** $p < 0.001$). However, treatment with piperine decreased unusually high LPO levels in group III and IV (## $p < 0.01$ and ### $p < 0.01$) (Figure 6).

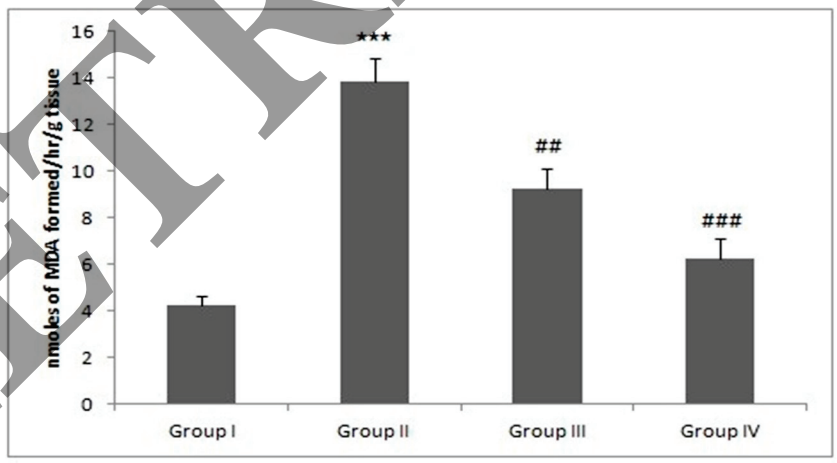


Figure 6. Piperine treatment alleviates MDA levels. In DMH-administered/tumor group-II, the MDA level was increased significantly (*** $p < 0.001$) as compared to control group. Treatment with piperine (30 and 60 mg/kg b. wt.) significantly alleviated MDA levels in group III (## $p < 0.01$) and group IV (### $p < 0.001$) as compared to group II ($n = 10$).

3.6. Piperine Treatment Alleviates Anti-Oxidant Armory

Piperine's effect on DMH-induced exhaustion of different antioxidants was studied. The results are shown in Table 1. There was a significant difference (*** $p < 0.001$) in the activity of antioxidants

between control and the DMH-administered group. However, piperine treatment reinstated the activity of all antioxidant enzymes to normal ($^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$) (Table 1).

Table 1. Effect of Piperine on Glutathione and Antioxidant Armory.

	Group I	Group II	Group III	Group IV
Reduced Glutathione (GSH; nmol mg protein)	214.72 ± 11.2	92.32 ± 8.31 ***	141.39 ± 11.3 $^{\#}$	197.52 ± 15.8 $^{\#\#\#}$
Oxidized Glutathione (GSSG; nmol mg protein)	31.02 ± 3.23	79.86 ± 3.03 ***	62.03 ± 4.91$^{\#}$	41.52 ± 5.02 $^{\#\#\#}$
GSH/GSSG Ratio	6.921 ± 0.82	1.156 ± 0.21 ***	2.279 ± 0.33 $^{\#}$	4.759 ± 0.61 $^{\#\#\#}$
GPx (nmol/ min/mg protein)	201.23 ± 17.1	82.43 ± 7.25 ***	160.28 ± 14.7 $^{\#\#}$	182.46 ± 17.3 $^{\#\#\#}$
GR (nmol min/min/mg protein	201.44 ± 19.5	84.25 ± 9.22 ***	139.12 ± 17.8 $^{\#\#}$	189.23 ± 20.9 $^{\#\#\#}$
SOD (units/ min/mg protein	10.63 ± 1.93	4.01 ± 0.41 ***	7.11 ± 0.73 $^{\#\#}$	9.03 ± 0.97 $^{\#\#\#}$
Catalase (nmol H_2O_2 consumed/min/mg protein	10.23 ± 1.62	4.82 ± 0.48 ***	5.94 ± 0.33 $^{\#}$	8.32 ± 0.72 $^{\#\#\#}$
H_2O_2 (nmol of H_2O_2 /g tissue)	185.2 ± 17.4	403.5 ± 32.1 ***	256.9 ± 21.3 $^{\#\#}$	205.7 ± 19.3 $^{\#\#\#}$

Group-I: Control; Group-II: DMH-administered group (20 mg/kg BW); Group-III: DMH rats treated with Piperine (30 mg/kg b.wt.); Group-IV: DMH rats treated with Piperine (60 mg/kg b.wt.). Data are represented as mean ± S.E.M ($n = 10$).

3.7. Piperine Treatment Mitigates H_2O_2 Levels

H_2O_2 levels in colon tissue in group II increased significantly (*** $p < 0.001$) when compared with group I. However, treatment with piperine in groups III and IV caused a marked decrease in H_2O_2 at both doses ($^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.01$) (Table 1) ($n = 10$).

3.8. Piperine Treatment Regulates pNF-κB

There was an increase in pNF-κB expression in group II when compared with group I. However, Piperine treatment to group III and group IV animals decreased pNF-κB expression significantly, inhibiting pNF-κB and its downstream elements (Figure 7).

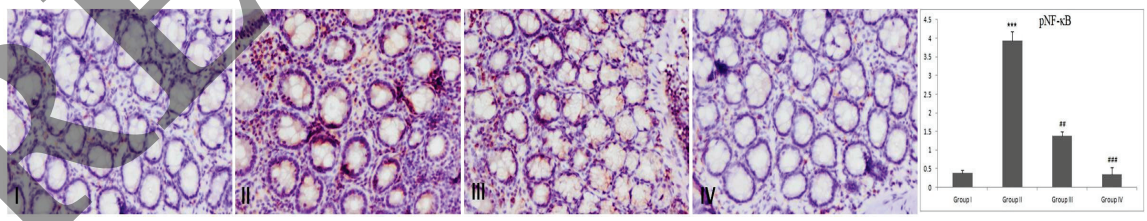


Figure 7. Effect of piperine treatment on pNF-κB expression. Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of pNF-κB and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has more pNF-κB immunopositive staining, as indicated by brown color, as compared to control group I, while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV reduced pNF-κB immunopositive staining as compared to group II ($n = 10$). Piperine significantly downregulated pNF-κB in group III and IV, respectively, ($^{\#\#}$ $p < 0.01$) and ($^{\#\#\#}$ $p < 0.001$), when compared with DMH-administered group-II ($n = 10$). Original magnification: 40×.

3.9. Piperine Treatment Mitigates Cox-2 and Inflammatory Mediators

Levels of inflammatory cytokines like TNF-α, PGE-2, IL-6 and Cox-2 were also increased significantly in the DMH-treated group II in comparison to untreated control group I (*** $p < 0.001$). However, Piperine treatment in groups III and IV attenuated TNF-α, PGE-2, and IL-6 and Cox-2 levels significantly (Table 2) at both the doses ($^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$), respectively (Table 2).

Table 2. Effect of Piperine on Serum Levels of Inflammatory Cytokines (IL-6, TNF-α, Cox-2, iNOS and PGE-2).

	Group-I	Group-II	Group-III	Group-IV
IL-6 (pg/mL)	834.12 ± 42.34	2023.32 ± 187.4 ***	1342.07 ± 146.6 $^{\#}$	1054.83 ± 94.50 $^{\#\#\#}$
TNF-α (pg/mL)	522.83 ± 40.27	1443.62 ± 138.2 ***	987.83 ± 105. $^{\#}$	774.31 ± 55.49 $^{\#\#}$
Cox-2 (pg/mL)	921.62 ± 63.11	1998.23 ± 92.3 ***	1532.13 ± 144.1 $^{\#\#}$	1098.23 ± 68.70 $^{\#\#\#}$
iNOS (pg/mL)	774.52 ± 87.63	1638.65 ± 106.1 ***	1310.22 ± 119.4 $^{\#\#}$	897.35 ± 77.42 $^{\#\#\#}$
PGE-2 (pg/mL)	814.89 ± 99.10	1547.86 ± 165.2 ***	1211.17 ± 113.4 $^{\#\#}$	998.64 ± 94.71 $^{\#\#\#}$

Group-I: normal control; Group-II: DMH-administered group (20 mg/kg BW); Group-III: DMH rats treated with Piperine (30 mg/kg b.wt.); Group-IV: DMH rats treated with Piperine (60 mg/kg b.wt.). Data are represented as mean of 10 rats ± S.E.M.

3.10. Piperine Treatment Mitigates NO and iNOS Production

There was a significant rise in NO and iNOS production in group II as compared with the group I animals (*** $p < 0.001$). However, treatment with Piperine effectively reduced NO and iNOS formation in group III and IV when compared with group II ($^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$) (Figure 8 and Table 2).

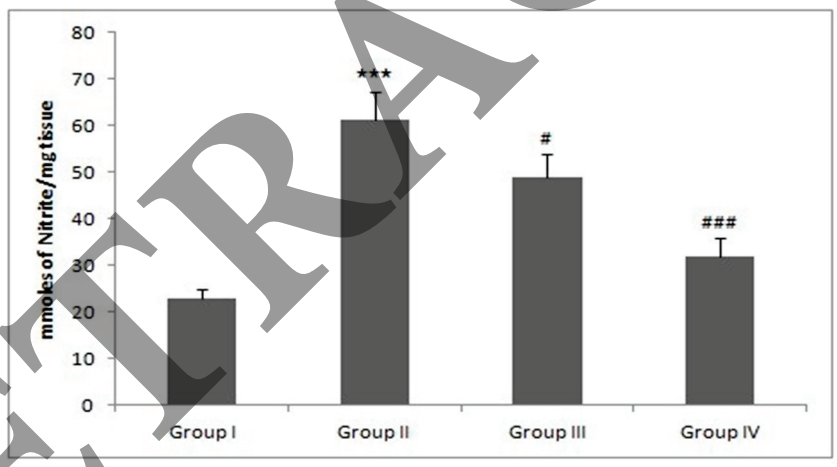


Figure 8. Effect of piperine and DMH-administered group on nitrite levels. In DMH administered group-II, the nitrite levels were significantly increased (*** $p < 0.001$) as compared to control group-I. Treatment with piperine significantly (30 and 60 mg/kg b. wt.) attenuated nitrite levels in group III ($^{\#}$ $p < 0.05$) and group IV ($^{\#\#\#}$ $p < 0.001$) as compared to group-II ($n = 10$).

3.11. Piperine Treatment Mitigates MPO Production

There was a significant rise in MPO production in group II as compared with the group I animals (*** $p < 0.001$). However, treatment with Piperine effectively reduced MPO formation in groups III and IV when compared with group II ($^{\#\#}$ $p < 0.01$) (Figure 9).

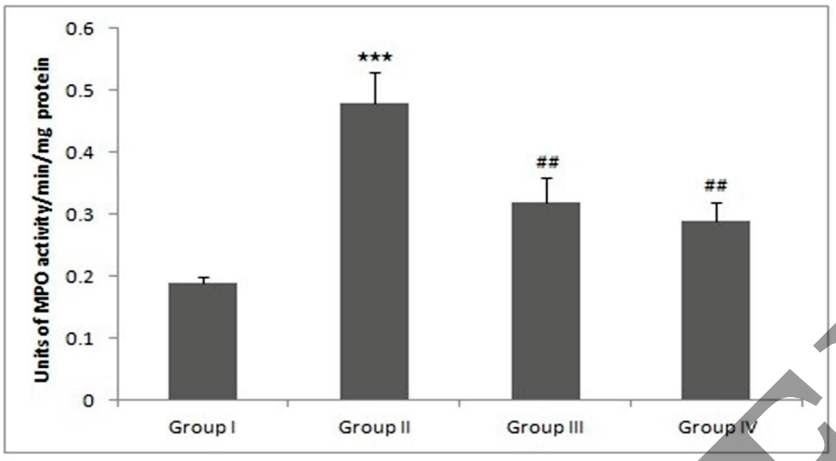


Figure 9. Effect of piperine and DMH-administered group on MPO levels. In DMH-administered group-II, the MPO levels were significantly increased (*** $p < 0.001$) as compared to control group-I. Treatment with piperine significantly (30 and 60 mg/kg b. wt.) attenuated MPO levels in group III ($^{\#\#}$ $p < 0.05$) and group IV ($^{\#\#}$ $p < 0.01$) as compared to group-II ($n = 10$).

3.12. Piperine Treatment Attenuates Mucin Staining in Colonic Tissue

There is negligible blue staining in control group, which indicates mucin integration as compared to the DMH-administered group. However, piperine treatment decreased disintegration of the mucous layer in groups III and IV (Figure 10).

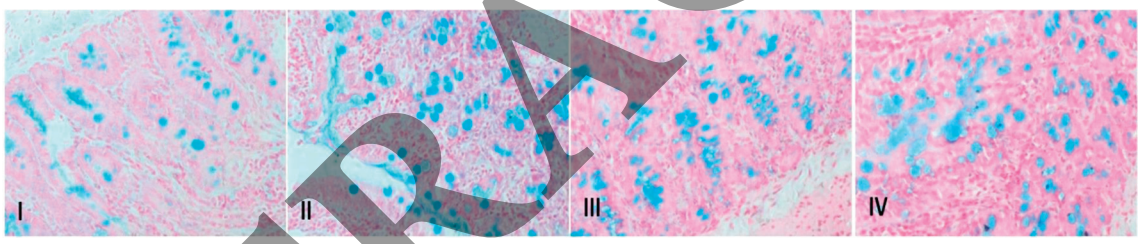


Figure 10. Photomicrographs showing mucin staining. There is decrease in mucin in mucous layer in DMH administered group, which is depicted by blue staining, when compared with control. Treatment with piperine increased mucous layer, as we can see from the lesser appearance of blue staining pattern in III and IV slides, representing groups III and IV ($n = 10$).

3.13. Effect of Piperine on the Colon Histology

Histological evaluation revealed that group I rats showed normal histological architecture with no signs of visible malformation/anomaly (Figure 11). There was disintegration of histoarchitecture like irregular colonic crypts, goblet cells disintegration, penetration of inflammatory cells, erosion of the mucous membrane, and gross inflammation was observed in DMH-administered rats. However, treatment with piperine restored cryptic architecture and goblet cell integration. Piperine also repealed adenoma formation and intrusion of inflammatory cells, hence regulating inflammation, which forms the basis of carcinogenesis (Figure 11).

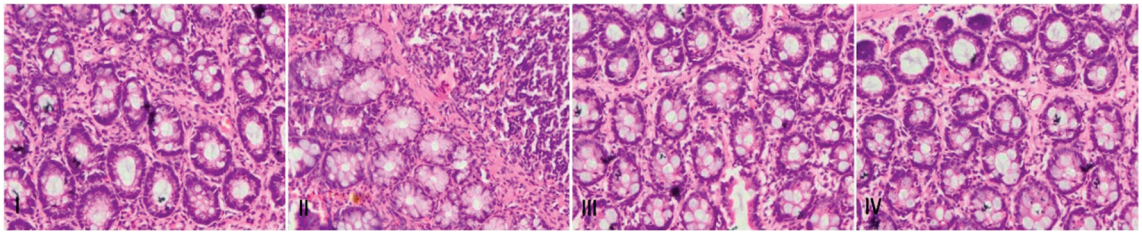


Figure 11. Effect of Piperine treatment on DMH-administered pathological changes in rat colon tissues. Photomicrographs of H&E staining of histological sections of colon tissue depicting different experimental groups: group-I indicate normal histo-architecture of colon sections. Group-II shows extensive disintegration of normal architecture in DMH administered group. In groups III and IV piperine treatment showed protection against piperine-induced pathological changes. Both the doses of piperine maintained the integrity of mucous membrane, goblet cells and colonic crypts (n = 10), magnification: 40×.

4. Discussion

Colon cancer process involves varied steps starting from pathological modifications extending from disconnected microscopic mucosal lesions of ACF and MDF to progression of tumor. Likewise, oxidative stress and inflammation are critical in the pathogenesis of cancer growth and promotion at molecular level. DMH was used as a carcinogen in the present study which mimics the human colon cancer pathology and does not only rely on extrapolating data from in vitro models due to the intricate complexity of genetic and epigenetic processes taking place inside humans which contribute to colon cancer. Hence, piperine was tested to find its anti-cancer efficacy at molecular, cellular and morphological levels, which have shown anti-colon cancer activity in vitro previously [6–10].

We first studied the influence of piperine on biomarkers of colon cancer. CEA is one of the most used tumour markers with predictive implication in early stages of colon cancer. It mediates adhesion intercellularly, promotes cell accretion, and controls immunity and signalling. Consequently, it shows tumour invasiveness and metastatic activity [29]. We did an investigation of the same biomarker in our experimental colon cancer study as well. It is not astonishing that CEA level was raised in DMH administered group significantly, as reports suggest that CEA is elevated in gastric and colon cancer. Nevertheless, the CEA level was decreased by the piperine treatment, possibly due to its anti-cancerous activities, resulting in the prevention of DMH-induced colon carcinogenesis. We also studied ACF and MDF, considerable pre-cancerous lesions found in colorectal cancer in carcinogen-administered rats as well in high-risk human cases. Because of their similarities in genotypic and phenotypic descriptions in animal and humans, ACF and MDF are standard biomarkers to detect and analyse pathogenesis of colon carcinogenesis at an early stage. The development of ACF and MDF due to DMH administration in experimental rodents was also used as a short-term model to investigate the protective mechanisma of organic compounds [15]. We found that piperine reduced ACF and MDF scores in group III and IV rats, respectively, when compared with the tumor group, which implies a role of piperine in inhibiting early events of colon carcinogenesis, which has been supported by earlier studies [30].

Next, we studied a robust signalling pathway, namely 2 (Nrf-2)/Keap-1), against oxidative stress for cellular protection. Nrf-2, a protective transcription factor, controls downstream antioxidant machinery or detoxification system molecules found in the cells. The instigation of Nrf-2 during the initial stage of inflammation-mediated tissue damage impedes the formation of proinflammatory modulators like pro-inflammatory cytokines, chemokines, and cell adhesion molecules [31]. The development and destruction of Nrf-2 are in balance under normal physiological conditions. However, activation of Nrf-2 is blocked by Keap-1 protein under stress conditions. Upon activation and dissociation of Keap-1, Nrf-2 accrues and binds with the antioxidant responsive elements (AREs) via translocating into the nucleus in the promoter region to activate the transcription and defensive activities of downstream antioxidant machinery like HO-1 and glutathione-dependent enzymes. In the current study, Nrf-2 expression was

diminished in DMH administered animals and treatment with piperine upregulated Nrf-2 expression and further activated regulatory enzymes downstream, as reported previously [32]. This was further validated and supported by a study where in Nrf-2- (−/−) mice were additionally vulnerable to dextran sulfate sodium (DSS)-induced colitis [33]. This demonstrated that Nrf-2 activation is an auxiliary therapy, possibly useful for colitis and colon cancer patients. The augmented sternness of colonic damage in DMH group animals may be correlated with a decrease in phase II detoxification enzymes, as reported previously [34]. In Nrf-2-deficient mice, proinflammatory modulators like interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) were prominently elevated as compared with the colon tissues of wild-type Nrf-2 mice [33,35]. Moreover, Nrf-2-deficient (Nrf-2−/ −) mice are at an increased risk of having various cancers like stomach, CRC and skin because they are increasingly vulnerable to oxidative injury-instigated ailments and DNA damage caused by chemicals as compared to wild-type mice. Khor et al. testified that Nrf-2 knockout mice with azoxymethane and DSS administration showed greater tumor incidence (80% versus 29%, respectively) as well as an augmentation of inflammatory markers like Cox-2, 5-lipoxygenase, PGE-2, and leukotriene levels in colon as compared with azoxymethane and DSS-treated wild-type (WT) mice [36]. In the present study, there was decreased expression of Nrf-2 and HO-1 in DMH group animals. However, treatment with piperine increased Nrf-2 and HO-1 expression in treatment groups. Thereby, alleviating ROS, and hence DNA damage, resulting in the protection of cells against potentially harmful entities may be the fundamental process through which Nrf-2 shields against chemical-induced carcinogenesis [37].

Keap-1 is a protein that keeps Nrf-2 in an inhibited state in cytoplasm. Dissociation and amendment of Nrf-2–Keap-1 complex is necessary for the transfer of Nrf-2 in the nucleus, which leads to the activation of Nrf-2–ARE-dependent signalling, and numerous cell transduction pathways. ARE inducers like piperine treatment downregulate Keap1 level in treatment groups as compared to the DMH group, and due to this, Nrf-2 translocation occurs, activating the downstream pathway. Various pathological events induce stress and HO-1 is a crucial protein that helps in cellular adaptation. It has been found to be a potent beneficial target for various oxidant and inflammatory diseases. The HO enzyme family provides carbon monoxide (CO) and free iron (Fe^2) by breaking down heme to biliverdin and bilirubin. Both bilirubin and CO may shield molecules against oxidative injury via a reduction in superoxide anions and lipid peroxidation. Additionally, reports indicate pro-apoptotic and anti-proliferative activities of HO-1 in prostate, breast and oral cancer, although the exact process of the act is unknown [14,38].

Nrf-2 is also found to control several phase II detoxification molecules like HO-1, NQO1), glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT) and glutamate-cysteine ligase (GCL) [39]. NQO1 is an important target of Nrf-2, is involved in detoxification process, and its upregulation thwarts the ability to amplify IL-1β and TNF-α [36]. In the present study, we found downregulation in HO-1 and Keap-1 in DMH group. However, treatment with piperine upregulated expression of HO-1 and Keap-1 at both the doses, signifying activation of Nrf-2. Nonetheless, activated Nrf-2 triggers ARE resulting in the activation of anti-inflammatory and anti-oxidant responses, activating protective responses in cells, as reported previously [40].

Oxidative stress and chronic inflammation are two of the life-threatening features intricately associated both in the commencement and progression of cancerous growth by modulating tumor microenvironment which, in ROS, acts as a secondary messenger to dysregulate various signaling pathways. The deficit of antioxidant machinery or detoxification system enzymes, as well as up-surging in ROS and RNS, is detrimental to colonic tissue homeostasis. We found that there was an increase in ROS production in the DMH-administered group as compared to control group. Treatment with piperine attenuated ROS levels in both groups, as reported previously [39]. MDA is a hallmark of oxidative stress produced by ROS and is a carcinogenic agent attributing to the formation of cancers in humans. We observed augmented levels of MDA in DMH-induced CRC as reported previously [41]. Piperine treatment repressed the production of MDA levels, which may be due to the alkaloids present in piperine scavenging free radicals due to its potent anti-oxidant activity attenuating the MDA level

in treatment groups. Enzymatic and non-enzymatic antioxidants are the primary defenders against cytotoxic oxygen radicals by scavenging intermediates of oxygen reduction [42]. Some anti-oxidants like SOD and CAT protect against lipid peroxidation in tissues by directly eliminating reactive oxygen metabolites like superoxide ($O_2^{2\bullet}$) and hydroxyl ions ($OH^{\bullet}$), providing one of the most efficient defensive mechanisms in the biological system against diseases [43]. CAT converts H_2O_2 to H_2O and O_2, hence averts oxidative injury. In the present study, SOD and CAT activities were diminished in DMH-induced CRC, whereas these activities were enhanced after the administration of piperine, which may be because of its free-redox-trapping activity [11]. GSH constitutes the major non-protein thiol in mammalian cells, which helps in many cellular activities like the regulation of protein synthesis. GSH acts as a substrate for many xenobiotic and free radical elimination reactions. Augmented GSH may stimulate other GSH-dependent enzymes like GPx, which has four selenium cofactors that catalyse the breakdown of H_2O_2 and organic hydroperoxides [41]. GR is another glutathione-restoring enzyme that catalyses oxidized glutathione (GSSG) to GSH by the oxidation of NADH to NAD [44]. In the current study, GSH was diminished and GSSG increased in DMH group in eliminating free radicals, hence the levels of reduced GSH were exhausted. However, piperine supplementation increased GSH levels and decreased GSSG in treatment groups due to its anti-oxidative potential. Moreover, the detoxification of xenobiotics, carcinogens, free radicals and peroxides occurs primarily through GPx and GR by conjugating xenobiotics with GSH, resulting in cellular protection against mutagen-induced toxicity. In the present study, there were decreased activities of GSH and GSH-dependent enzymes like GPx and GR in DMH-induced rats, whereas treatment with pieprine increased the antioxidant armory, which may be because of its free radical scavenging potential [45]. Earlier reports recommend that alterations in redox balance and signaling are hallmarks of the initiation and promotion of carcinogenesis and resistance to treatment [46]. Hence, agents that upregulate antioxidant enzyme machinery like CAT, SOD, GR, GPx, which inactivate ROS, have a massive potential to avert the initiation and promotion of cancer, as observed in the current study. Therefore, the upregulation of antioxidant machinery and the preclusion of DMH-induced colon carcinogenesis by prophylactic treatment of piperine may possibly be due to the presence of alkaloids which resulted in boosting endogenous anti-oxidant machinery because of its anti-oxidant, anti-inflammatory and various pharmacological activities attributed to it [11,12,29,39,47].

In the instigation and development of cancer, a potential cross-talk between Nrf-2 and NF-κB pathways is recognized. One of the principle ways through which chronic inflammation leads to the formation of neoplasm/growth is by the production of ROS via inflammatory cells. This Nrf-2 intermediated anti-cancer response is accomplished by not only an increase in antioxidant armory, but, additionally confirmed by the repression of inflammatory pathway mediators smoothed through the NF-κB signaling pathway. Correspondingly, an upregulation of cytokines occurs due to Nrf-2 deficiency as a result of NF-κB activation or the activation of Nrf-2 attenuates NF-κB and downstream signalling, as reported previously [47,48]. Carini et al. reported that the dysregulation of ROS may alter DNA assembly, and consequentially modify proteins and lipids, and stimulate numerous stress-activated transcription elements including NF-κB, the formation of pro and anti-inflammatory cytokines which contribute to carcinogenesis via oxidative insults [38,49]. However, antioxidant agents like piperine impede IL-1beta, iNOS, Cox-2, NO, NF-κB production and, henceforth, carcinogenesis [40,50].

Chronic inflammation contributes to 25% of human cancers. One of the most important pathways involved in inflammation is NF-κB. NF-κB is a redox specific transcription factor which activates immune and cell detoxification systems, endorses the development of pro-inflammatory cytokines like TNF-α, IL-1, IL-6, and IL-8, and promotes tumor growth [51]. NF-κB's enhanced expression may be attributed to chronic inflammation followed by cyclic administration with carcinogen; DMH contributes to colon carcinogenesis. Other studies also decipher that colon cancer cell lines have remarkably abnormal NF-κB expression and lower IκB levels, showing that dysregulated NF-κB is a major contributor to colon cancer, which is in accordance with our results as well [52]. Our results further validate that the piperine inhibits NF-κB activity, as reported previously, and strongly recommend

that the bioactivity of piperine against colon carcinogenesis may possibly be due to the inactivation of NF-κB, which is further evident due to the inhibition of downstream pathway player proteins [11,53,54].

NF-κB has a critical connection amongst inflammation and cancer since it increases levels of tumor-stimulating cytokines downstream, like IL-6, TNF-α, PGE-2, Cox-, as well as survival genes such as Bcl-extra-large (Bcl-xL). Similar results were obtained in the current study. ROS is released by the activation of inflammatory cells, resulting in oxidative injury to DNA and proteins. There are cumulative indications which decipher that the chances of colon carcinogenesis increase by enhancement of pro-inflammatory cytokines like TNF-α, IL-1α, IL-1β and IL-6, which further increase the secretion of PGE-2, an inflammatory mediator. However, piperine treatment attenuated the levels of the above-mentioned pro-inflammatory cytokines, possibly by the inhibition of NF-κB and anti-inflammatory activity of piperine, as reported previously [54–56].

Cox-2 is triggered in inflammatory or hypoxic environments and is upregulated in various cancers, including colon cancer, unlike in normal cells, thus making it a potential therapeutic target. The major downstream mediator of Cox-2 is PGE-2, which enhances cellular growth and angiogenesis, obstructs apoptosis, augments invasiveness, and regulates immunosuppression in colonic mucosa. Additionally, enhanced Cox-2 expression is directly proportional to PGE-2, resulting in increased production of malondialdehydes, forming DNA adducts in colonic tissue, and quickens the process of carcinogenesis. In the current study, we found an increase in Cox-2 and hence PGE-2 in the DMH group, whereas treatment with piperine decreased Cox-2 and hence PGE-2 in both the groups, thereby precluding the process of carcinogenesis and not allowing its acceleration [56].

NO, an RNS, an inflammatory mediator, plays an essential role in colon tumorigenesis in both humans and investigational experiments. NO is formed by three isoforms of nitric oxide synthase (NOS), under normal physiological conditions. iNOS produces micromolar concentrations of NO which last for hours or days, which is a greater amount than in NO production by other isoforms of NOS, resulting in DNA damage, deficiency of DNA repair, cancerous growth proliferation and promotion. Consequently, tumor malignancy is enhanced by variations brought about by NO formation in cells by damage/mutation in DNA, dysregulating signalling pathways and instability in genome DNA repair pathways are negatively impacted by high NO concentrations, leading to carcinogenesis [9]. iNOS is produced by proinflammatory agents and the tumor micro-environment and does not depend on calcuim. Femia et al. reported that DMH-induced colorectal cancer has high pro-inflammatory enzyme iNOS expression [57]. We obtained similar results in our current study, which is supported by earlier findings. However, over-production of NO contributes by iNOS damaging DNA repair and promoting cancer growth. Therefore, mitigating iNOS, and hence NO, at early stages of colon cancer in humans can be beneficial strategy to diminish development of cancer. In addition, carcinogens may increase the activities of Cox-2 and iNOS on the mucosa of the colon, which also causes the promotion of cancer occurrence. Both Cox-2 and iNOS are upregulated by NF-κB. It was indicated that by reducing NF-κB expression, the occurrence of cancer can be reduced and decrease the induction of NOS. Thereby, decreasing NO further alleviates DNA damage and hence reduces cancer growth and promotion [10,11].

MPO is another enzyme found in neutrophils for H_2O_2 production. It has been reported that in the inflamed tissues, MPO level is directly proportional to the neutrophil concentration and infiltration. Thus, acute intestinal inflammation is evaluated by MPO measurement, which acts as a quantitative and sensitive assay. Inflammation is associated with oxidative stress and promotes tumor initiation and promotion. Chronic intestinal inflammation and colon cancer are related to each other. The present study found that the colon cancer group showed an elevation in the intestinal inflammatory markers like MPO and Cox-2 through the NF-κB-mediated response. Piperine treatment alleviated MPO levels through suppression of NF-κB, henceforth suppressing the infiltration of neutrophils and chronic inflammation leading to colon cancer [15,58].

The histology of colon tissue sections of control did not show any anomaly in crypt morphology and architecture, with no signs of inflammation. DMH group animals showed infiltration of inflammatory

cells, aberrant crypt formation, depletion of mucin, tissue necrosis, ulcer formation and cellular injury. However, Piperine treatment decreased hemorrhage formation, infiltration of inflammatory cells, edema in sub mucosal layers, and ulceration, as reported previously [59–62]. We discovered a similar pattern in the present study after treatment with piperine, as it restored the histo-architecture, similar to that of normal colon.

Therefore, the results of the current study indicate a novel bifunctional role of NF-κB suppression, i.e., deactivating the NF-κB modulated inflammatory pathways and in the upregulation of Nrf-2 facilitated anti-oxidative/anti-inflammatory pathways. Thus, Piperine looks like a substantially potential bioactive phyto-product with imminent scientific application in prophylactic treatment of colon carcinogenesis. However, the immunohistochemical results could be further explored through quantitative estimations which would further support/clarify our immunohistochemical, histological and biochemical data. However, in future studies, clarity and certainty about mechanisms could be revealed through how piperine impacts colon cancer at preclinical and clinical levels.

5. Conclusions

It is imperative to find innovative ways for effective treatment of colorectal cancer. Even though widespread improvement has been made in the treatment of colorectal cancer, yet millions of people die annually due to colon cancer worldwide. Nrf-2 and its downstream mediators protect cells from extracellular and intracellular oxidative damage and maintain redox homeostasis while NF-κB contributes to survival and resistance of colon cancer cells. We show that prophylactic treatment of piperine activates Nrf-2 pathway which triggers antioxidant response machinery mediators like HO-1, NQO-1 GSH, GR, GPx, CAT, SOD, scavenges ROS; decreases lipid peroxidation; blocks NF-κB and its associated downstream signalling molecules which include Cox-2, PGE-2, TNF-α, IL-6, NO, iNOS and other inflammatory mediators like NO, MPO. Our histological findings and pre-cancerous markers of colon further validate beneficial effects of piperine on alleviating DMH induced colon carcinogenesis. Hereafter, mitigation of Nrf-2/Keap-1 pathway and pre-cancerous events by piperine may possibly be a potential mechanism. Henceforth, piperine may be a promising molecule for the prophylactic treatment of colon carcinogenesis.

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Cytotoxicity and Pro-Apoptotic, Antioxidant and Anti-Inflammatory Activities of Geopropolis Produced by the Stingless Bee *Melipona fasciculata* Smith

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Abstract: Geopropolis is produced by some stingless bee species, such as *Melipona fasciculata* Smith, a native species from Brazil. This study aims to investigate the antioxidant and anti-inflammatory activities and cytotoxicity effects of geopropolis hydroethanolic extracts against lung (H460 and A549) and ovarian (A2780 and ES2) cancer cell lines and non-tumor (HUVEC) cell lines using chemical identification by LC/MS/MS analysis and in silico assays to determine which compounds are associated with bioactivity. The antioxidant activity of extracts and inhibitory activity against COX enzymes were assessed by in vitro assays; cytotoxicity effect was evaluated by the MTT assay; cell cycle was assessed by flow cytometry and apoptosis by Western blotting. The geopropolis extracts showed great radical scavenging potential, preferential inhibition of COX-2, decreased cancer cell viability, non-cytotoxic effects against the non-tumoral cell line, besides modulating the cell cycle and inducing cancer cell apoptosis through the activation of caspase-3 and PARP protein cleavage. The in silico study suggests that corilagin, typhaneoside, taraxerone and marsformosanone, identified by LC/MS/MS, can be associated with anti-inflammatory activity and cytotoxic effects. Thus, the current study suggests the potential of geopropolis concerning the research field of new pharmacological alternatives regarding cancer therapy.

Keywords: natural products; antitumor activity; new anticancer agents; apoptosis pathway; molecular docking; drug discovery

1. Introduction

Cancer arises from the gradual accumulation of genetic alterations that increase cell proliferation [1]. It is the second main cause of death and has been recognized as one of the major public health problems worldwide. In 2018 alone, over 18 million new cancer cases were reported and over 9.5 million deaths by cancer were recorded worldwide, according to GLOBOCAN [2].

The gold standard treatment consists of the use of chemical neoplastic agents such as alkylating agents, antimetabolites, topoisomerase inhibitors and mitotic inhibitors which, in many cases, no longer present encouraging results and result in severe side effects [3]. Therefore, the development of new alternative drugs exhibiting low toxicity, high efficiency and the ability to prevent cell proliferation and/or promote apoptosis has become the major focus of cancer therapy in recent years [4,5].

In addition to apoptosis-inducing drugs, anti-inflammatory agents selective for cyclooxygenase 2 enzyme (COX-2) are traditionally reported be an effective adjuvant strategy for cancer therapy. COX-2 is involved in several malignant neoplasm processes, such as in the promotion of apoptotic resistance and in the proliferation, angiogenesis, inflammation, invasion and metastasis of cancer cells. Therefore, the use of COX-2 inhibitors is significant in managing metastasis risk reduction attempts in cancer patients while also resulting in higher susceptibility of cancer cells to gold standard treatments, such as radio and chemotherapy, resulting in better treatment efficiency [6].

Currently, the search for natural products exhibiting potential in cancer therapy has become prominent [7,8]. Geopropolis, a natural product derived from stingless bees, is noteworthy among natural products, displaying the highest potential in this regard.

Geopropolis is produced by stingless bees, formed by resinous material from plants collected by the bees, salivary bee secretions, wax, and clay or soil [9]. *Melipona* (*Melikerria*) *fasciculata* Smith 1858 (*Apidae*, *Meliponini*) is a stingless bee species cultivated for centuries by the indigenous population and small producers of Baixada (flooded fields) and Cerrado (Brazilian savannah) areas in Maranhão, a northeastern Brazilian state, to produce honey, geopropolis, wax and pollen [10,11].

Several geopropolis biological properties have been reported, including antinociceptive and anti-inflammatory [12–14], immunomodulatory [15,16], antimicrobial [14,17,18], antileishmanial [19] and antioxidant activities [19–23].

The antitumoral activity of *M. fasciculata* geopropolis has been previously evaluated against canine osteosarcoma (OSA) cells and cytotoxic effects have been reported in human leukemia monocytic cell lines [24,25]. However, the cytotoxic action of *M. fasciculata* geopropolis in other tumor cell lines has not yet been investigated.

In this context, this research aimed to evaluate the antioxidant, anti-inflammatory and cytotoxic activities of a hydroethanolic geopropolis extract of produced by *M. fasciculata*, identifying its chemical composition and correlating the identified compounds with detected biological activities through in silico assays and, finally, to contribute to the bioprospecting of new products exhibiting antitumor activity.

2. Materials and Methods

2.1. Geopropolis Samples

Two geopropolis samples of *M. fasciculata* Smith were collected in April 2018 being taken directly from the internal parts of a beehives located in meliponary in Viana (03°13′13″ S and 45°00′13″ W) and Pinheiro (02°31′17″ S and 45°04′57″ W) municipalities in the "Baixada" (flooded fields area, Brazil) from Maranhão State, northeast Brazil. After collection, the geopropolis samples were separated, identified, stored in a sterile recipient and kept at 4 °C until preparation of extract and further analysis. As determined by Brazilian legislation for research that uses the Brazil's genetic heritage, this research is registered on National System of Genetic Heritage Management and Associated Traditional Knowledge (SISGEN) under code ABCEA59.

2.2. Extraction of Samples

The in natura geopropolis samples were processed as described by our research group in Dutra et al. [21]. The geopropolis was triturated until powder (200 g) and were individually extracted by maceration with 70% ethanol/water (70:30, *v*/*v*) for 6 days at a solid:solvent ratio of 1 to 5 (*w*/*v*), with solvent renewal after 72 h. The resulting product from extractions was combined, filtered, concentrated in a rotary evaporator under vacuum at 40 °C, and lyophilized, obtaining the hydroethanolic geopropolis extract (EHGV) (Viana sample) and EHGP (Pinheiro sample) and kept refrigerated until their use.

2.3. Determination of Antioxidant Activity

2.3.1. DPPH• Radical Scavenging Activity

The antioxidant activity of hydroethanolic geopropolis extracts was evaluated by using the DPPH• free radical scavenging assay as described by Brand–Willians [26] with modifications as described by our research group in Dutra et al. [21]. The samples of geopropolis extracts were solubilized on methanol at concentrations (30–480 μg/mL) and added to a methanol solution of DPPH• (40.0 μg/mL). After 30 min of reaction at room temperature in the dark, the absorbance of each solution was read at 517 nm in a Lambda 35 UV–Vis spectrophotometer (PerkinElmer, Inc., Waltham, MA, USA). Methanol ACS was used as blank, and DPPH• solution was used as negative control. Trolox® (positive control) standards were treated under the same conditions as the samples. The percent inhibition was calculated using the formula

$$\text{DPPH• scavenging activity (\%)} = 100 - (A_{sample} - A_{blank}) \times 100/A_{control}$$

where A_{sample} = absorbance of the sample after 30 min of reaction, A_{blank} = absorbance of the blank, and $A_{control}$ = absorbance of the control.

The results were expressed as inhibitory concentration at 50% (IC_{50}). All experiments were performed in triplicate.

2.3.2. Ferric Reducing Antioxidant Power Assay (FRAP)

The method described by Benzie et al. [27] with some modifications as described by our research group in Dutra et al. [21] was used to assessment the antioxidant activity based on iron reduction using the FRAP assay, measuring the ferric-reducing ability of a sample in acid medium (pH 3.6) through the formation of an intense blue color as the ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex due reduction to the ferrous (Fe^{2+}) form. The samples of hydroethanolic geopropolis extracts were solubilized in methanol at different concentrations (12.5–200 μg/mL). The absorbance of the reaction mixture was read at 593 nm in a Lambda 35 UV–Vis spectrophotometer (PerkinElmer, Inc., USA) using FRAP solution as a blank. The calibration curve was constructed using different concentrations of $FeSO_4 \cdot 7H_2O$ (0–2000 μM) ($R^2 = 0.9892$), and the results are expressed as millimoles of Fe^{2+} per gram of sample. Trolox® standard was set as positive control. The results were expressed as millimoles of Fe^{2+} per gram of sample. All experiments were done in triplicate.

2.3.3. ABTS•$^+$ Assay

The ABTS•$^+$ method (2,2′-azinobis-3-ethylbenzotiazoline-6-sulfonic acid) was carried out as described by Re et al. [28] with modifications by our research group in Lopes et al. [11]. For formation of the ABTS radical, the 7 mM ABTS•$^+$ solution was mixed with 2.45 mM of potassium persulfate solution. This mixture was maintained in a dark room for 16 h for the complete oxidation of ABTS and the generation of the highly stable chromophore cation radical 2,2′-azino-bis-(3 ethylbenzothiazoline-6-sulfonic acid) (ABTS•$^+$). The radical was diluted in 70% ethanol/water (70:30, *v*/*v*) to an absorbance of 0.700 ± 0.020 as read at 734 nm. Readings were performed

by reacting 20–1000 μg/mL of hydroethanolic geopropolis extracts with the ABTS•$^+$ solution. All studies were performed at least in triplicate, monitoring the decrease in absorbance for 6 min; reported results correspond to the % of remaining chromophores compared to conditions in the absence of antioxidants. The IC_{50} values were determined to each sample, using the formula

$$\text{Scavenging ability (\%)} = (1 - A_{sample}/A_{blank}) \times 100.$$

2.4. In Vitro COX Inhibition

The assay was performed according to the manufacturer's recommendations (COX Colorimetric Inhibitor Screening Assay Kit—Item No. 701050—Cayman Chemical®, Ann Arbor, MI, USA), as previously described by our research group in Lopes et al. [11]. Using 96-well microplates, the geopropolis extracts were evaluated at three different concentrations (2, 10 and 50 μg/mL) against COX-1 and COX-2 isoforms. After reagents and plates preparations following the manufacturer's kit data sheet, the colorimetric analysis was performed, using arachidonic acid as the substrate of the COX-catalyzed enzyme reaction, and the plates were read at 590 nm.

2.5. Cell Culture and Morphological Analysis

A panel of four cancer cell lines (H460, A549, ES2 and A2780) and non-cancer cell (HUVEC) were selected for this study. Non-small cell lung carcinoma (NSCLC) cell lines A549 and H460, and ovarian cancer cell lines ES2 and A2780 were maintained in RPMI-1640 (Gibco, New York, NY, USA) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) (Gibco, New York, NY, USA), 1% (*v*/*v*) stabilized with penicillin solution (100 units/mL) and streptomycin (100 μg/mL) (Gibco, New York, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Human umbilical vein endothelial cells (HUVEC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York, NY, USA) supplemented with 10% (*v*/*v*) FBS (Gibco, New York, NY, USA), 1% (*v*/*v*) stabilized penicillin solution (100 units/mL) and streptomycin (100 μg/mL) (Gibco, New York, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Short tandem repeat (STR) analysis was performed in all four cell lines to confirm cell line identity. The cell morphology was examined using an inverted Zeiss Observer Z1 microscope and images were captured using Axio-Vision Rel. 4.8 software (Carl Zeiss, Jena, Germany).

2.6. Cytotoxicity Activity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to measure the cytotoxic effects of (EHGV and EHGP) and cisplatin (CDDP) (LIBBS, SP, Brazil) on human cancer cell lines and non-tumoral cells. Stock solutions of 1000 mg/mL of EHGV and EHGP were dissolved in dimethyl sulfoxide (DMSO). Desired concentrations of each drug were prepared by dilution with culture medium before use. The cell lines were seeded (1×10^4 cells/well) at 96-well plates and after 24 h, were treated with EHGV and EHGP for 48 h and 72 h. Cells were subsequently incubated with 20 μL of MTT solution (5 mg/mL) for 4 h. The plates were then centrifuged at 450× *g* and to allow solubilization of the formazan crystals. Then, 100 μL of DMSO was added to each well and spectrophotometric absorbance reading at a wavelength of 538 nm was performed using Flex-Station 3 (Molecular Devices Corporation, San Jose, CA, USA). As negative control for the experiments, we used the cells without treatment. We also treated cells with vehicle control (10% DMSO *v*/*v*). All experiments were performed in quadruplicates. The half maximal cell growth inhibitory concentrations (GI_{50}) values were estimated using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, CA, USA).

2.7. Cell Cycle and DNA Content Analysis by Flow Cytometry

The DNA content and cell cycle distribution of tumor cells treated with EHGV were determined by flow cytometry after propidium iodide staining. Cells were seeded onto 6-well plates, and after 24 h, were treated with EHGV. The cells were subsequently collected and washed in phosphate-buffered saline (PBS) followed by incubation with Nicoletti buffer (0.1% NP-40 (*w*/*v*), 0.1% sodium citrate (*w*/*v*),

200 μg/mL RNase and propidium iodide 50 μg/mL) at 4 °C for 30 min in the dark [29]. Doublets and debris were identified and excluded. The samples were acquired at the low flow rate and at least 20,000 cells were counted for each analysis. The distribution of cells in each phase of the cell cycle was displayed as histograms. The stained samples were analyzed with FACSCalibur flow cytometry (BD, San Jose, CA, USA) and the results were analyzed using FlowJo software (FlowJo, LLC, Glendale, CA, USA).

2.8. Western Blotting

To analyze apoptosis, cells were trypsinized and plated into a six-well plate, and after 24 h, treated with EHGV (15.62 and 31.25 μg/mL) and CDDP (10 μM) for 48 h. Then, cells were lysed in 2× radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors. Protein concentration was determined using the DC Protein Assay kit (BioRad, Hercules, CA, USA). Equivalent amounts of protein (50 μg) were separated by SDS-PAGE. The gels were then transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% skim milk. Membranes were then incubated with primary antibodies against cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA, USA), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA, USA). Before blocking, the membranes were incubated with 1% glutaraldehyde for 30 min for the analysis of cleaved caspase-3 expression [30]. Detection was visualized with the ECL prime reagent (GE Healthcare, Sao Paulo, Brazil) and the images were then captured on a ChemiDoc Imaging system (Biorad, Hercules, CA, USA) using Image Lab software (Biorad, Hercules, CA, USA).

2.9. LC–ESI/IT–MS/MS Analysis

The EHGV and EHGP were analyzed by LC–ESI/IT–MS/MS (LC–20AD Shimadzu, Kyoto, Japan) and a Phenomenex Luna C–18 (250 × 4.6 mm, 5 μm) column at 25 °C was used. The mobile phase used was Milli-Q water (Millipore) with 0.1% formic acid (eluent A) and methanol (eluent B). Elution was performed on a linear gradient of 0 min—10% B; 1–40 min—100% B; 40–60 min—100% B. EHGV and EHGP were diluted in methanol and 0.1% Milli-Q water of formic acid at the final concentration of 30 mg/mL and filtered through a nylon filter (0.22 μm, Allcrom Sao Paulo, Sao Paulo, Brazil). The samples volume injected into the system was 10 μL, with a flow rate of 1 mL/min and UV–Vis detection at 254 nm. The LC was coupled to a mass spectrometer (Amazon Speed ETD, Bruker, MA, USA) equipped with electrospray ionization (ESI) and an ion trap (IT) type analyzer in negative mode under the following conditions: 4.5 kV capillary voltage, capillary temperature of 325 °C entrainment gas (N_2) flow 12 L/min, nitrogen nebulizer at a pressure of 27 psi. The acquisition range was *m*/*z* 100–1000, with two or more events. The compounds were identified on the basis of the molecular ion mass fragmentation.

2.10. In Silico Assay

2.10.1. Predictive Models and Theoretical Calculations

The metabolites identified in EHGV were schematically designed in 3D models on GaussView 5.0.8 [31] and had their geometric, electronic and vibrational properties optimized with Gaussian 09 [32] using the density functional theory (DFT) method, combining the hybrid functional B3LYP and the basis set 6-31 ++ G (d, p).

2.10.2. Molecular Docking (MD)

All MD protocol utilized Autodock Vina [33]. The structure of the human cyclo-oxygenase 2 (COX-2) (PDB ID 5F19) and nuclear factor kappa-B (NF-κB) NEMO/IKKβ association (PDB ID 3BRV) and ligands were prepared for MD with AutoDock Tools, version 1.5.7 [34]. Docking methodology described in literature were used [35] with some modifications [36,37]. Gasteiger partial charges

were calculated after addition of all hydrogens both in ligands, COX-2 and NEMO/IKKβ association structures. Non-polar hydrogens from COX-2, NEMO/IKK IKKβ and EHGV metabolites were subsequently merged. The dimensions of cubic box in the *x*-, *y*- and *z*-axes were 30 × 30 × 30. Grid box was centered on oxygen atom from residues Arg120 from COX-2 and Glu89 of NEMO domain. In addition to visual inspection, the initial coordinates of interaction complexes were chosen based on the criterion of better docking conformation of the lowest energy score.

2.11. Statistical Analysis

Statistical analyzes between groups were performed by analysis of variance (ANOVA) followed by Tukey test. The results that presented probability of occurrence of null hypothesis lower than 5% ($p < 0.05$) were considered statistically significant. Statistical analysis was performed using GraphPad Prism 8 software.

3. Results

3.1. Antioxidant Activity

The results show that EHGV had the highest antioxidant activity with DPPH IC_{50} (76.16 ± 1.05 μg/mL). In the FRAP assay, EHGV also showed a higher ferric reduction (2.91 ± 0.12 mmol Fe^{2+}/g) compared to EHGP (1.10 ± 0.25 mmol Fe^{2+}/g). Regarding the ABTS•$^{+}$ IC_{50}, EHGV was also the sample with high antioxidant activity (13.28 ± 0.11 μg/mL) (Table 1). All differences found were statically significant ($p < 0.05$).

Table 1. Antioxidant activity (DPPH•, FRAP, $ABTS^{\bullet+}$) of hydroethanolic geopropolis extract produced by *Melipona fasciculata* Smith.

Sample	DPPH• IC_{50} (μg/mL)	FRAP (mmol Fe^{2+}/g)	$ABTS^{+}$ IC_{50} (μg/mL)
EHGV	76.16 ± 1.05 [a]	2.91 ± 0.12 [a]	13.28 ± 0.11 [a]
EHGP	265.91 ± 0.29 [b]	1.10 ± 0.25 [b]	58.94 ± 0.09 [b]
Trolox	3.01 ± 0.47	8.41 ± 0.28	3.69 ± 0.63

Values represent the mean of triplicate measurements ± standard deviation. Different letters in the same column indicate a significant difference (Tukey $p < 0.05$). EHGV—hydroethanolic geopropolis extract of *M. fasciculata* from Viana city, Maranhão State, Brazil; EHGP—hydroethanolic geopropolis extract of *M. fasciculata* from Pinheiro city, Maranhão State, Brazil; DPPH•: 2.2-diphenyl-1-picrylhydrazyl radical; FRAP: ferric reducing antioxidant power; ABTS•+: 2.2′-azinobis-3-ethylbenzotiazoline-6-sulfonic acid.

3.2. COX Inhibition Assay

Both EHGV and EHGP were tested for their ability to inhibit COX-1 and COX-2 enzymes. EHGV showed high COX-2 inhibitory activity (44% and 61% at 10 and 50 μg/mL, respectively) with less than 5% inhibition of COX-1 at 50 μg/mL. EHGP shown a minor potential for COX-2 inhibition at the same concentrations and had COX-1 inhibition next to 10% (Figure 1).

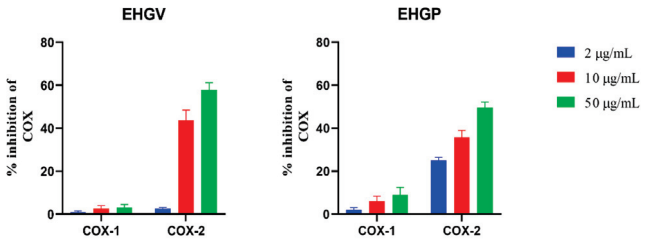

Figure 1. Percentual in vitro inhibition of COX-1 and 2 produced by hydroethanolic geopropolis extracts produced by *M. fasciculata* stingless bee was obtained in Viana (EHGV) and Pinheiro (EHGP) cities, Maranhão State, Northeast of Brazil.

3.3. Cell Culture and Morphological Analysis

To assess the antitumoral activity of EHGV, we firstly treated A2780 ovarian cancer cells with 15.62, 31.25 and 62.5 μg/mL EHGV for 48 h. Morphological differences were observed between the EHGV-treated, control cells and cells treated with 10% DMSO (*v*/*v*) (vehicle) (Figure 2A,B vs. Figure 2D–F). It is possible to observe that after treatment with EHGV, the cells become rounded and shrunken and detached themselves from the substrate. These morphological changes were absent in control cells and cells treated with vehicle (Figure 2). Morphological changes are valuable for determining the preliminary potential of anticancer activity of EHGV. As positive control, we used CDDP (10 μM) at a clinically relevant concentration [38] (Figure 2C).

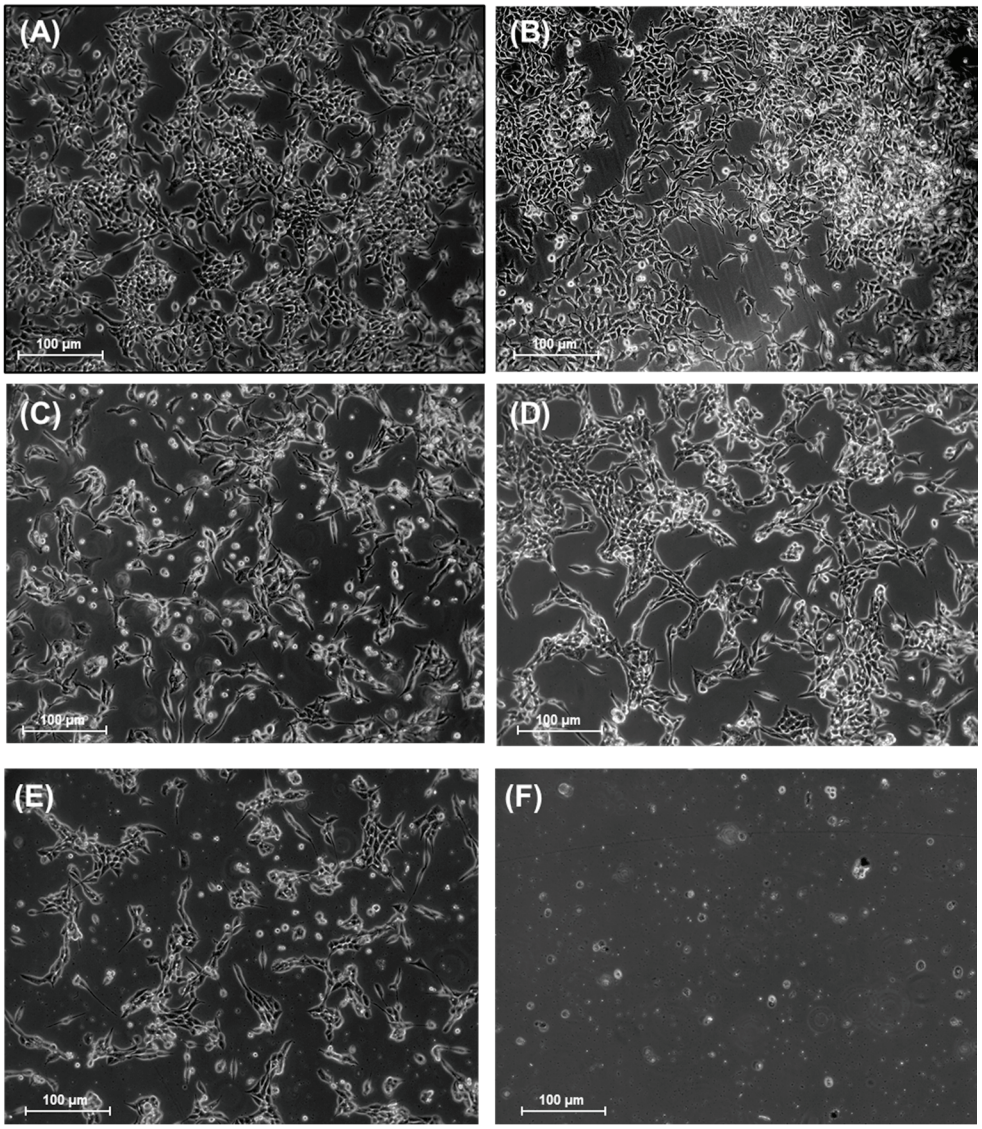

Figure 2. Analysis of morphological changes in A2780 tumor cells treated with EHGV. (**A**) Untreated A2780 control cells; (**B**) A2780 cells treated with vehicle (10% (*v*/*v*) DMSO); (**C**) A2780 cells treated with CDDP 10 μM; (**D**–**F**) A2780 cells treated with 15.62, 31.25 and 62.5 μg/mL EHGV, respectively. Cells were exposed to various concentrations of EHGV, CDDP and DMSO vehicle control and morphological changes were observed following 48 h of treatment. The cells were photographed (magnification 10×) with Axio-Vision Rel. 4.8 software. Scale bar = 100 μm.

3.4. Cytotoxic Activity

Then, we assessed the cytotoxic activity of EHGV and EHGP in lung cancer (A549 and H460), ovarian cancer (ES2 and A2780) and non-tumoral (HUVEC) cell lines treated with increasing concentrations of EHGV and EHGP for 48 and 72 h.

The results obtained through the MTT assay revealed that the extracts decreased the percentage of cell viability for ovarian (A2780 and ES2) and lung (A549 and H460) cancer cells in a dose- and time-dependent manner. The EHGV and EHGP extracts showed greater cytotoxicity in the highest concentrations evaluated and in the longer incubation time with the extract. EHGV extract demonstrated high cytotoxic effect in the 48 and 72 h time compared to EHGP. Among the seven concentrations used, it was observed that from 31.25 µg/mL, the EHGV extract already demonstrated the capacity to inhibit percentage growth (Figure 3).

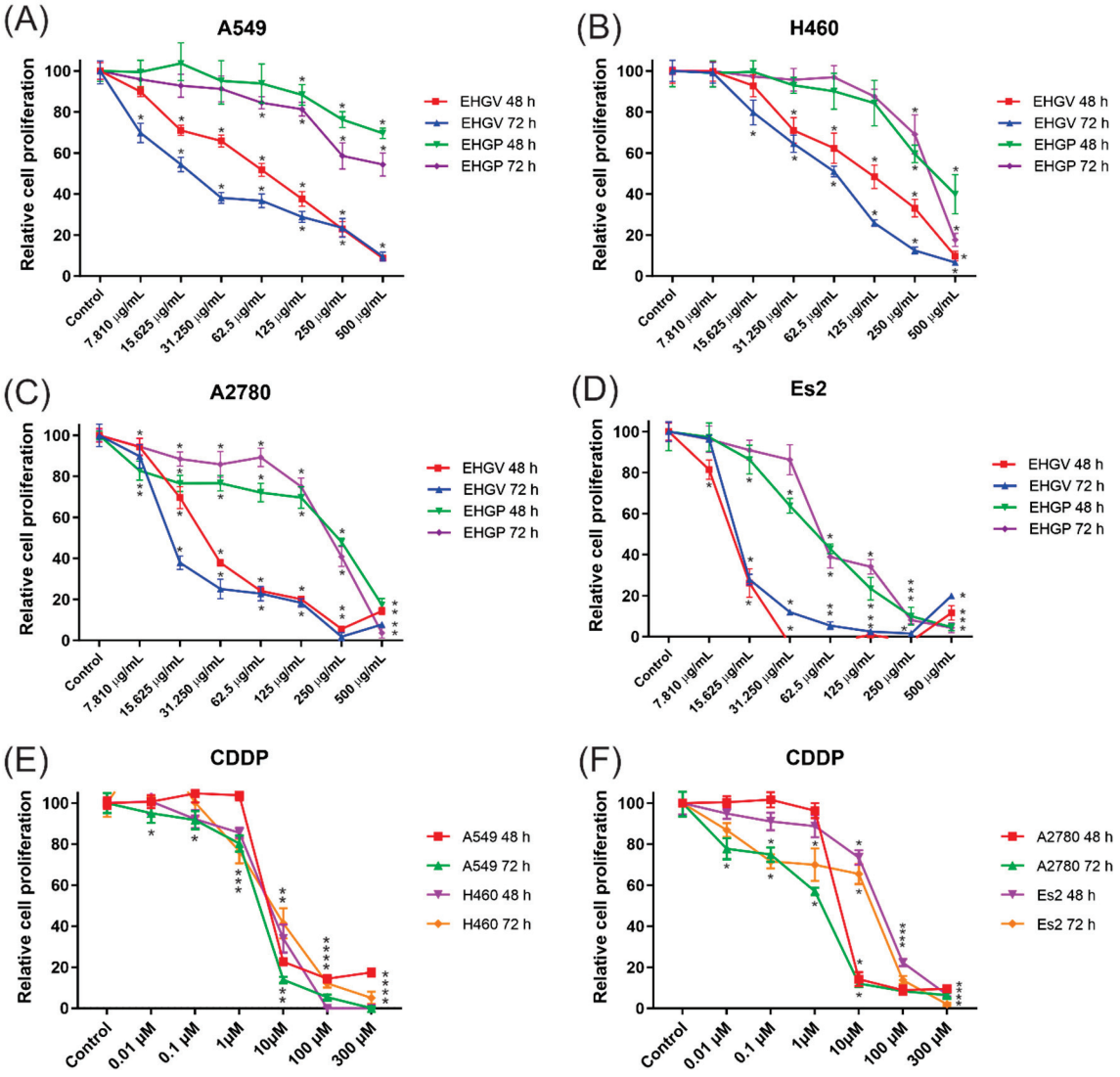

Figure 3. Effects of EHGV and EHGP in A549 (**A**), H460 (**B**), A2780 (**C**), Es2 (**D**) and CDDP (**E**,**F**) in four cancer cell lines at 48 and 72 h with statistical results. 2-way ANOVA with Tukey post-test. (* indicates $p \leq 0.05$; vs. control).

Due to the high cytotoxicity for normal cells and numerous side effects caused by most of the traditional chemotherapy drugs used nowadays, we evaluated the cytotoxicity effects of EHGV and EHGP extracts against non-malignant HUVEC cells. As observed in Figure 4A, treatments with EHGV

and EHGP for 48 and 72 h showed a non-significant influence on the cell viability of HUVEC cells. EHGV and EHGP at the most elevated concentration (500 µg/mL) for 72 h barely maintained cell viability in more than 80% (80.85 ± 5.90% and 89.54 ± 6.49%, respectively) of HUVEC cells. Importantly, when HUVEC cells were treated with CDDP, an extensively used chemotherapeutic drug for lung and ovarian cancer, we observed a decrease in cell viability in 48 and 72 h. We highlight the treatment with 10 µM, considered a clinically relevant concentration of CDDP, that markedly reduced cell viability close to 80% (only 17.61 ± 6.20% and 13.87 ± 4.01% of cell viability) in 48 and 72 h, respectively (Figure 4B). Collectively, these findings show that the present extracts exerted significant cytotoxic effects on cancer cells while showing low toxicity against non-malignant HUVEC cell lines.

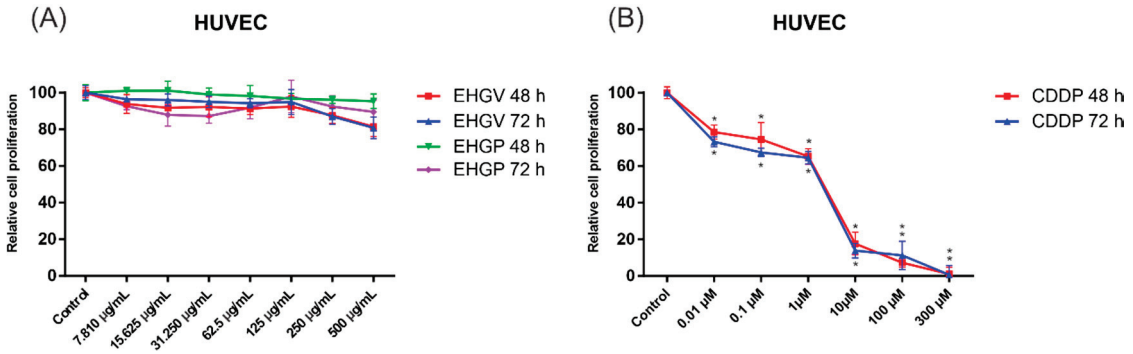

Figure 4. Effects of EHGV and EHGP (**A**) and CDDP (**B**) in non-tumor cells, HUVEC at 48 and 72 h with statistical results. 2-way ANOVA with Tukey post-test (* indicates $p \leq 0.05$; vs. control).

The values for cell growth inhibition (GI_{50}) of the EHGP and EHGV extracts were determined individually by the MTT assay over 48 and 72 h in the A2780, Es2, A549, H460 and HUVEC cell lines and are shown in Table 2. The results show that the EHGV extract significantly inhibited A2780 and A549 and was the most potent extract with an GI_{50} value of 16.92 µg/mL for A2780 and 22.64 µg/mL for A549 (Table 2).

Table 2. Cell growth inhibition (GI_{50}) in µg/mL of EHGP and EHGV for inhibition of cell proliferation in cancer cell lines (A2780, ES2, A549, H460) and normal cells (HUVEC).

Time	Sample	Cell Line				
		A2780	**ES2**	**H460**	**A549**	**HUVEC**
48 h	EHGV	313.6 µg/mL	133.1 µg/mL	105.4 µg/mL	105.4 µg/mL	113,300 µg/mL
	EHGP	177.4 µg/mL	51.4 µg/mL	360.6 µg/mL	1122 µg/mL	40,650 µg/mL
72 h	EHGV	16.92 µg/mL	137.7 µg/mL	56.51 µg/mL	22.64 µg/mL	5537 µg/mL
	EHGP	196 µg/mL	64.83 µg/mL	311.9 µg/mL	551.1 µg/mL	indeterminate

EHGV = hydroethanolic geopropolis extract of *M. fasciculata* from Viana city, Maranhão State, Brazil; EHGP = hydroethanolic geopropolis extract of *M. fasciculata* from Pinheiro city, Maranhão State, Brazil; GI_{50} values calculated by non-linear regression equation log (inhibitor) versus response—variable slope by the MTT assay. Concentration required to inhibit cell growth by 50% as determined by the dose response curve. Values are expressed as mean ± standard deviation of cytotoxicity assays (n = 4).

3.5. Cell Cycle Analysis by Flow Cytometry and Analysis of Apoptosis by Western Blot

To explore the possible mechanisms underlying EHGV cytotoxicity in cancer cells, we analyzed cell cycle distribution and induction of apoptosis thought of cleaved caspase-3 and cleaved PARP in A2780 cells. Cell cycle distribution was analyzed by flow cytometry after a 48 h exposure to EHGV (15.65 and 31.25 µg/mL). EHGV treatment increased cells in S-phase compared with control cells treated with vehicle (Figure 5A). Additionally, treatment with EHGV at 31.25 µg/mL led to the accumulation of

small DNA fragments in the sub-G1 phase (hypodiploid peak) in A2780 cells compared to control cells (5% vs. 9.79%). Interestingly, the treatment with CDDP, a drug currently employed in the treatment of ovarian cancer, showed the equivalent results in A2780 cells (8%) (Figure 5A).

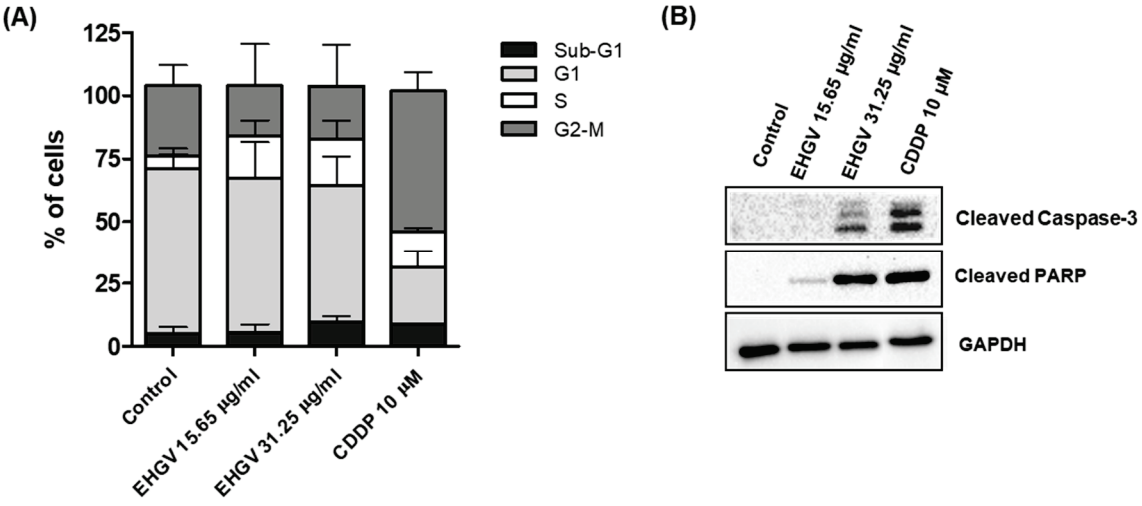

Figure 5. Analysis of effect of EHGV on cell cycle phase distribution and Western blot analysis of apoptosis-related proteins in A2780 cells treated with EHGV (15.65 and 31.25 µg/mL) and CDDP (10 µM) for 48 h. (**A**) Distribution of cells in sub-G1, G1, S or G2/M phases of cell cycle in A2780 cells treated with EHGV (15.65 and 31.25 µg/mL), CDDP (10 µM) and vehicle (control) for 48 h. (**B**) Western blot analysis of cleaved caspase-3 and cleaved PARP in A2780 cells treated with EHGV (15.65 and 31.25 µg/mL), CDDP (10 µM) and vehicle (control) for 48 h. GAPDH was used as loading control.

Intrigued with the low proportion of cells in the sub-G1 phase (considered as apoptotic cells) and the impressive results in decreasing the percentage of cell viability in ovarian cancer cells (Figure 3), we next verified apoptosis-related proteins by Western blot. Cleavage of caspase-3 (17 kDa subunit) and PARP (89 kDa subunit) to their active forms is an important event in cancer cell apoptosis. As shown in Figure 5B, treatment with EHGV for 48 h increased the expression of cleaved caspase-3 and cleaved PARP in a dose-dependent manner. Additionally, A2780 cells treated with CDDP 10 µM for 48 h also showed an increase in these apoptosis-related proteins.

3.6. LC–ESI/IT–MS/MS Analysis

Tables 3 and 4 summarize the list of identified compounds in the classes of glycosylated flavonoids, triterpenes, triterpenoid saponins, hydrolyzable tannins, anthraquinones and catechins, their retention time, molecular weight, molecular ions $[M - H]^-$ and main ions of the products obtained by LC–ESI/IT–MS/MS for the 23 peaks of fragmentation of EHGV and EHGP.

Table 3. Compounds identified in the hydroethanolic geopropolis extract produced by *M. fasciculata* stingless bee from Viana city, Maranhão State, Brazil, by *LC–ESI/IT–MS/MS.*

Compound	RT (min)	Identification	MW	$[M - H]^-$ (*m*/*z*)	MS/MS Fragments (*m*/*z*)
1	2.8	gluconic acid	196	195	128; 177
2	3.1	corilagin	634	633	615; 484
3	15.9	taraxerone	424	423	304; 334; 406
4	18.2	myricetin-3-*O*-α-arabinopyranoside	450	449	430; 359; 329
5	19.1	prunin	434	433	313
6	20.5	dipterocarpol	443	442	209; 165

Table 3. *Cont.*

Compound	RT (min)	Identification	MW	[M − H]⁻ (*m*/*z*)	MS/MS Fragments (*m*/*z*)
7	24	taxifolin 7-*O*-rhamnoside	450	449	405
8	24.7	isoschaftoside	564	563	548; 298
9	25.3	marsformosanone	422	421	377; 333; 297; 214; 179; 157
10	32	β-amyrin	427	426	232
11	40	typhaneoside	770	769	375; 331
12	44.5	3-[xyl]-28-glc-phytolaccagenin	826	825	403; 360

RT, retention time; MW, molecular weight; [M − H]⁻ molecular ion.

Table 4. Compounds identified in the hydroethanolic geopropolis extract produced by *M. fasciculata* stingless bee from Pinheiro city, Maranhão State, Brazil, by *LC–ESI/IT–MS/MS*.

Compound	RT (min)	Identification	MW	[M − H]⁻ (*m*/*z*)	MS/MS Fragments (*m*/*z*)
1	2.8	gluconic acid	196	195	128; 177
2	16	taraxerone	424	423	304; 334; 364; 406
3	17.8	dihydroquercetin-*C*-glycoside	450	449	431; 359; 329; 287; 303
4	18.2	dihydroquercetin-*C*-glycoside isomer	450	449	430; 359; 329
5	19.1	narigenin-*C*-glycoside	434	433	313
6	20.2	narigenin-*C*-glycoside isomer	434	433	415; 313
7	21	vitexin-*O*-galate	584	583	169; 313; 932; 537
8	22.5	pinobanksin glycosilated	436	435	270; 151; 341; 391
9	22,9	dihydroquercetin 3-*O*-ramnoside	450	449	303; 405
10	33.8	xantholaccaic acid A	521	520	262; 357; 419; 458; 502; 542
11	42.5	gallocatequin-xylose	438	437	305; 357; 393; 437

RT, retention time; MW, molecular weight; [M − H]⁻ molecular ion.

3.7. In Silico Study

In the molecular docking study, all metabolites identified in EHGV were used. All compounds showed very satisfactory affinity parameters to both COX-2 and NF-κB structures. On COX-2, high parameter values were found for corilagin, typhaneoside and β-amyrin, with values for free binding energies of −9.3, −8.8 and −8.7 kcal/mol, respectively. Regarding NF-κB, taraxerone, marsformosanone and β-amyrin had parameters indicating high interaction, with free binding energies of −8.4, −7.7, −7.4 kcal/mol, respectively. The results of the free binding energy parameters of EHGV metabolites are shown in Table 5.

Table 5. Free binding energies obtained by molecular docking of the compounds identified in EHGV.

COX-2		NF-κB	
Ligand	**ΔG_{bind} (kcal/mol)**	**Ligand**	**ΔG_{bind} (kcal/mol)**
corilagin	−9.3	taraxerone	−8.4
typhaneoside	−8.8	marsformosanone	−7.7
β-amyrin	−8.7	β-amyrin	−7.4
isoschaftoside	−8.6	dipterocarpol	−6.9
3-[xyl]-28-glc-phytolaccagenin	−8.5	3-[xyl]-28-glc-phytolaccagenin	−6.9
marsformosanone	−8.5	prunin	−6.8
taraxerone	−8.3	corilagin	−6.6
prunin	−8.0	typhaneoside	−6.5
myricetin-3-*O*-α-arabinopyranoside	−7.9	isoschaftoside	−6.4
dipterocarpol	−7.7	myricetin-3-*O*-α-arabinopyranoside	−6.3
taxifolin 7-*O*-rhamnoside	−7.6	taxifolin 7-*O*-rhamnoside	−6.0

The docking results showed that corilagin, typhaneoside and β-amyrin have hydrogen bonds and van der Waals interactions with residues from COX's catalytical site triad (Arg120, Thr385 and Glu524) and neighboring residues. It is also shown that the taraxerone, marsformosanone and β-amyrin performs van der Waals interactions with residues Glu89, Lys90, Leu93, Met94 and Phe97 (NEMO domain) and Glu729, Gln730, Ser733 and Phe734 (IKKβ domain). The spatial conformation from ligands obtained by the molecular docking study is shown on Figure 6.

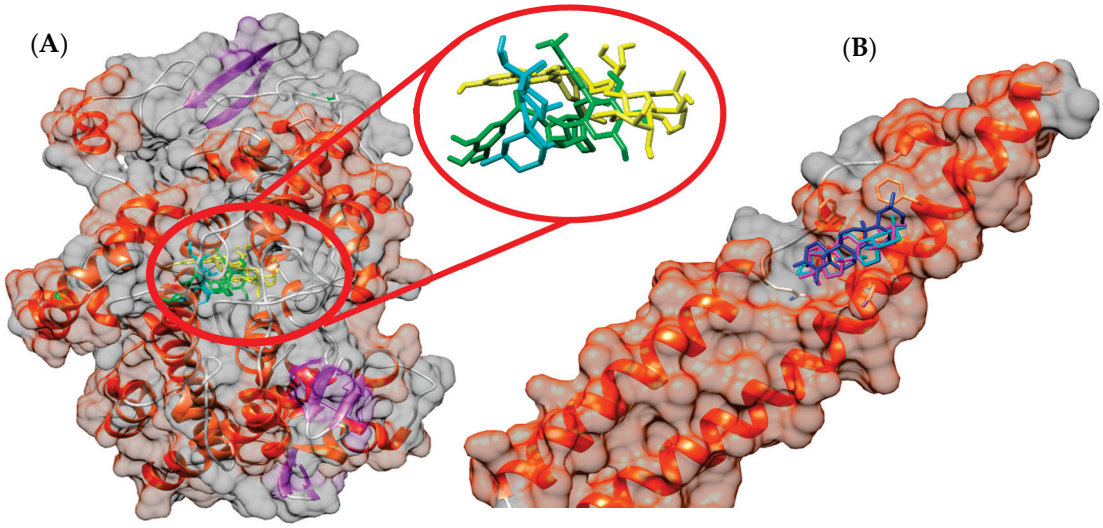

Figure 6. Spatial conformations obtained by molecular docking of corilagin (in green), typhaneoside (in yellow) and β-amyrin (in cyan) on COX-2 active site (**A**) and conformations of taraxerone (in blue), marsformosanone (in magenta) and β-amyrin (in cyan) on NEMO/IKK β structure (**B**).

4. Discussion

The findings reported herein indicate that EHGV and EHGP exhibit the ability to reduce ferric ions to ferrous ions (FRAP) and free radical scavenging activity (DPPH• and ABTS•$^{+}$), suggesting significant antioxidant activity (Table 1). These results are in agreement with previously reported findings from stingless bee geopropolis [21,39,40].

Several types of oxidative damage at the cellular level are avoided in biomolecules such as lipoproteins and/or DNA, due to the presence of antioxidants. In the absence of these compounds, the oxidative stress produced by an increased production of reactive oxygen species (ROS) is responsible for triggering a complex and wide cascade of biochemical events harmful to organisms and associated to several pathological and disease processes, including cancer [41,42]. In addition, the interactions of

antioxidant compounds with ROS through the elimination of free radicals are implicated in reducing conditions triggered by oxidative stress, such as cancer and other inflammatory processes [43].

Considering the antioxidant activity of *M. fasciculata* geopropolis extracts, COX inhibition potential and antitumor activity were evaluated by an in vitro cytotoxicity assay, where anti-inflammatory and antioxidant activities can be associated to antitumor activity [43,44]. Due to its higher antioxidant activity, EHGV also exhibited major inhibitory activity, inhibiting 61% of COX-2 and only 5% of COX-1 at 50 μg/mL (Figure 1). EHGV also demonstrated higher cytotoxic effect, decreasing cell viability in ovarian (A2780 and ES2) and lung (A549 and H460) cancer cells (Figure 3). Extracts exhibiting antioxidant properties commonly act as enzyme inhibitors, i.e., COX, xanthine oxidase, lipoxygenase, phospholipase A_2 and others. The prostaglandin metabolism mediated by COX plays a fundamental role in inflammatory processes and is important in carcinogenesis, tumor differentiation, tumor growth and in suppressing tumor immunity, contributing to cancer immunotherapy resistance to several types of tumors [6,45,46]. In addition, a possible carcinogenesis inhibition mechanism has been reported for COX-2 inhibition since this enzyme plays an important role in the activation of local growth factors that lead to neovascularization, inflammation and carcinogenesis [47]. Thus, products that inhibit COX-2, such as *M. fasciculata* geopropolis, exhibit the potential to integrate future preventive and therapeutic anticancer strategies.

Considering the importance of lung and ovarian cancers, both resulting in high mortality rates, usually detected in advanced stages and exhibiting significant chemoresistance [2,48], two lung cancer cell lines (A549 and H460) and two ovarian cancer cell lines (ES2 and A2780) with different genetic backgrounds were selected for antitumoral activity evaluation of *M. fasciculata* hydroethanolic geopropolis extracts.

Several morphological changes in A2780 ovarian cancer cells treated with EHGV were observed, especially at the highest exposed concentration (62.5 μg/mL) (Figure 2). EHGV-treated cells became rounded and shrunken, exhibiting decreased density and detaching themselves from the substrate. These features are suggestive of EHGV-induced cell death mediated through apoptosis. Changes in cell morphology were less prominent when treated with clinically relevant concentrations of CDDP, routinely used in lung and ovarian cancer treatment [48].

Furthermore, the MTT assay was used to measure the cytotoxic effects of the extracts in lung and ovarian cancer cell lines. Our findings indicate that EHGV demonstrated higher cytotoxic effects at 48 and 72 h compared to EHGP in all cancer cell lines (Figure 3).

Drug discovery of new cytotoxic agents that explores differences between cancerous and normal cells continues to be a public health demand. Therefore, anticancer agents are expected to exhibit minimum effects on non-tumor cells. For this reason, we also evaluated extract cytotoxicity in non-malignant HUVEC cell lines. No significant toxic effects were observed, reinforcing the safety of the geopropolis extract and emphasizing its ability to inhibit cell proliferation, promoting its antitumor activity (Figure 4). Our investigations regarding the toxic effects of the extract corroborate Barboza et al. [49], who evaluated acute EHGV and EHGP toxicity in a zebrafish toxicity model and reported very low toxicity.

Previous studies have reported that *M. fasciculata* geopropolis exerts cytotoxic effects in a human leukemia monocytic cell line with significative decreases in cell viability at high concentrations (50 and 100 μg/mL) [25]. Additionally, Cinegaglia et al. [24] reported that *M. fasciculata* geopropolis also exhibits cytotoxic activity against canine osteosarcoma (OSA) cells. In another study, geopropolis produced by *Melipona mondury* Smith exhibited significant antiproliferative activities against various tumor cell lines (mouse melanoma, B16-F10, human hepatocellular carcinoma, HepG2, human promyelocytic leukemia, HL-60 and human chronic myelocytic leukemia K562) and no cytotoxic effects against non-tumor cells [14].

According to the USA's National Cancer Institute (NCI), crude extracts with $GI_{50} < 30$ μg/mL in the preliminary assay are considered promising cytotoxic agents against neoplastic cells [50,51]. Therefore, EHGV is a promising product due to a GI_{50} value of 16.92 μg/mL in A2780 cancer cell lines

and 22.64 μg/mL in A549 cancer cell lines (Table 2). Regarding the four-human cancer-derived cell lines, the A2780 ovarian cancer cell line was selected to screen EHGV's ability to induce apoptosis (programmed cell death) and its impact on the cell cycle due to a higher GI_{50} value of 16.92 μg/mL for A2780. The A2780 cell line was treated with EHGV at two concentrations (15.65 and 31.25 μg/mL) for 48 h and then analyzed by flow cytometry. EHGV-treated A2780 cells displayed an increased percentage of cells in the S-phase compared with control cells treated with the vehicle. As mentioned previously, CDDP is routinely employed in the treatment of ovarian cancer and exhibited similar effects concerning an increased number of cells in the sub-G1 phase compared to the EHGV (31.25 μg/mL) treatment in A2780 cells (Figure 5A). It is important to emphasize that no studies have characterized cell cycle modulation after geopropolis extract exposure in cancer cells.

Apoptosis, a form of programmed cell death, involves the activation, expression, and regulation of various proteins [52]. Clinically, the main goal in cancer therapy is cancer cell death. Caspase-3 is a central apoptosis effector, catalyzing the specific cleavage of many key cellular proteins, like nuclear protein poly (ADP-ribose) polymerase (PARP) [53,54]. The results of the present study also indicate that EHGV dose-dependently enhances A2780 cell apoptosis after 48 h of treatment by increasing the expression of cleaved caspase-3 and cleaved PARP (Figure 5B).

PARP plays an essential role in several cellular process, such as maintenance of genomic stability, DNA repair and apoptosis [55], and is a target of caspase-3 protease activity. Caspase-3 cleaves PARP into two fragments (89 and 24 kDa) during apoptosis. This cleavage is considered a useful hallmark of cell apoptosis [56]. As shown in Figure 5B, EHGV activates caspase-3 cleavage and, consequently, reduces proteins levels throughout the total PARP length in EHGV-treated cells. This can be accompanied by increased PARP expression, an apoptosis characteristic [57].

Following the positive results of decreased cell viability in lung and ovarian cancer cells, chemical composition characterization was performed. The LC–ESI/IT–MS/MS analysis indicates a chemical composition similarity between the two extracts related to glycosylated flavonoids and triterpenes (taraxerone). Triterpenes ursane (marsformosanone), oleanane (β-amyrin) and dammaren (dipterocarpol) skeleton and glycosylated triterpene saponin (3-[Xyl]-28-Glc-phytolaccagenin) were identified in EHGV, which were not detected in EHGP (Tables 3 and 4). These findings corroborate studies concerning the chemical composition of *M. fasciculata* geopropolis from different areas, due to the predominance of substances of the polyphenolic classes (hydrolysable tannins and flavonoids) and triterpenes [19,21,40,58].

Yam-Puc et al. [59] identified thirteen pentacyclic triterpenes in a chloroform–methanol–propolis extract from *Melipona beecheii* including marsformosanone, taraxerone and β-amyrin, which were also identified in EHGV. Besides taraxerone, two other pentacyclic triterpenoid (marsformosanone and β-amyrin) and one dammaren triterpene (dipterocarpol) and one glycosylated triterpene saponin (3-[Xyl]-28-Glc-phytolaccagenin) were also identified in EHGV. The triterpenes marsformosanone and dipterocarpol and 3-[Xyl]-28-Glc-phytolaccagenin, a triterpene saponin, were identified for the first time in *Melipona fasciculata* geopropolis. Triterpenoids, in general, are commonly attributed to inhibition of NF-κB activation and signal transduction, cell proliferation, apoptosis, angiogenesis and mitochondrial dysfunction [8].

According to the EHGV anti-inflammatory and antitumor activity results, which suggest that COX-2 receptor and NF-κB play a role in these effects, molecular docking of the compounds identified in the EHGV against these targets was also performed (Figure 6).

The molecular docking results suggest that corilagin, a hydrolysable tannin, typhaneoside, glycosylated flavonol, and the triterpene β-amyrin exhibit the best affinity parameters to COX-2 structure (Table 5). Corilagin exhibited greater interaction and its antitumor and anti-inflammatory potential is described in the literature, which may act in suppressing COX-2 expression at the gene and protein levels, demonstrating that this molecule may inhibit the inflammatory process [60–62]. No records concerning typhaneoside COX inhibitor potential are available, but is recognized that this molecule can regulate IL-6 and TNF-α [63] and promote cell proliferation and decrease NO levels

in HUVEC cells [64], suggesting anti-inflammatory activity and no cytotoxic effects. β-Amyrin also displays antitumor effects against HepG2 liver carcinoma cells, causing apoptosis, cell cycle disruption and activation of the JNK and p38 signaling pathways [65]. β-Amyrin reduces the gene expression of TNF-α, IL-1β, IL-6, PGE2, COX-2 [66] and exhibits high inhibition of PGE2 and IL-6 secretion and NF-κB activation in a concentration-dependent manner, being a promising molecule for the treatment of various inflammatory disorders [67].

COX-2 is the main enzyme acting in prostaglandin (PGH2) production through the conversion of arachidonic acid. This prostaglandin can later be converted into different prostaglandins such as PGE_2, PGD_2, $PGF_{2\alpha}$, and also into thromboxane A_2. These inflammatory prostanoids are closely associated to rapid and disordered tumor growth, characteristic of malignant neoplasms, since they promote cell division, metastasis and angiogenesis, in addition to inhibiting cell apoptosis [68,69]. Thus, the fact that EHGV displays a preference for COX-2 inhibition is directly correlated to the antitumor capacity reported herein.

Regarding the NF-κB receptor, taraxerone, marsformosanone and β-amyrin exhibited the best affinity parameters in the molecular docking study, with their conformations as the best free binding energy in the same region (Table 5), interacting with important catalytic residues such as the Glu89 NEMO and Ser733 IKKβ domains [70]. Taraxerone, identified in both EHGP and EHGV, has been described in the literature as displaying antitumor potential, inhibiting cancer cell proliferation and colony formation in A549 lung cancer cells, revealing potent cytotoxic effects in a dose-dependent manner and characteristic of apoptosis [71]. Taraxerone has also been reported as an antioxidant and iNOS inhibitor [72]. No records regarding marsformosanone's potential as an NF-κB inhibitor agent are available, so this is the first study to suggest this activity. Pentacyclic triterpenes have been described as potential NF-κB signaling pathway inhibitors [73–75]. Laszczyk [76] described that triterpenes with a lupano, oleanan or ursano skeleton, including β-amyrin, display antitumor activity against different modes of action. β-Amyrin shows anti-inflammatory and anti-apoptotic effects on hepatic fibrosis in male rats [77]. Additionally, Ghante and Jamkhande [8] reported that triterpenoids display the ability to inhibit NF-κB activation.

NF-κB regulates the transcription of anti-apoptotic genes, contributing to cancer cell escape from apoptosis [78]. NF-κB inhibition in experimental studies has shown promising results in enhancing apoptosis and potentiating antitumor agent effects [79]. In addition, NF-κB is pivotal in inflammatory responses. Therefore, inhibition of the NF-κB signaling pathway exhibits potential therapeutic application in cancer and inflammatory diseases [80,81].

Thus, based on our EHGV result, an EHGV mechanism of action in ovarian cancer cells is suggested (Figure 7).

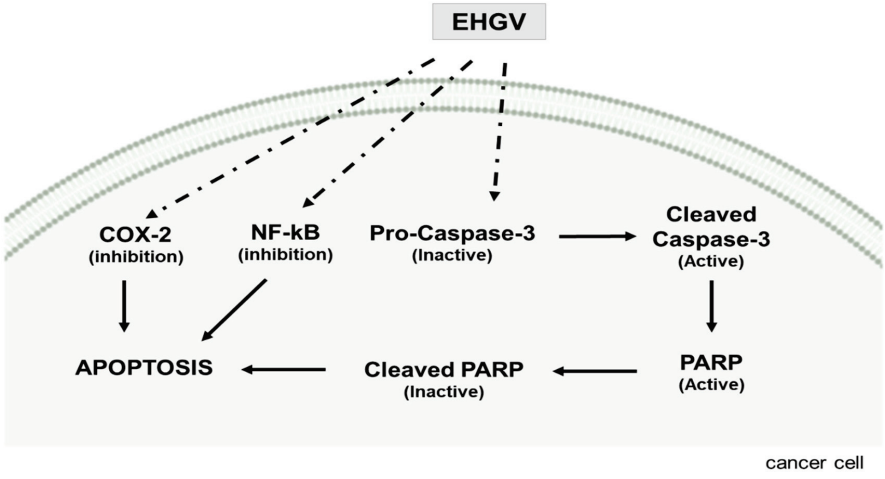

Figure 7. Proposed EHGV mechanism of action in ovarian cancer cells.

These findings reinforce our hypothesis that these molecules have the potential to become research targets for new drugs exhibiting anti-inflammatory and antitumor activities. Therefore, geopropolis may be a less toxic therapeutic alternative to be tested in the future in combination with monotherapy or polytherapy cancer treatment regimens.

5. Conclusions

The hydroethanolic geopropolis extract produced by *Melipona fasciculata* is composed of hydrolysable tannin, glycosylated flavonoids, anthraquinone, catechin, and triterpene substances which can be related to the antioxidant and anti-inflammatory activities and cytotoxic effects against A2780, ES2, A549, H460 cell lines. The extracts also have high preference for COX-2 inhibition, contributing effectively to antitumor activity. The in silico results, in concordance with our results from anti-inflammatory and antitumor activities, suggests that this activity can be due to COX-2 inhibition and NF-κB activation. Thus, we demonstrated for the first time that geopropolis produced by *M. fasciculata* has cytotoxic effects thought mediating apoptosis and cleaved caspase-3 activation in cancer cells, showing low toxicity against non-malignant HUVEC cell lines. We conclude that geopropolis is a natural product that exhibits anticancer properties that should be further evaluated in monotherapy or polytherapy schemes to improve chemotherapy–antitumor responses and long-term benefits in cancer patients.

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Antifungal Activity and Phytochemical Screening of Vernonia amygdalina Extract against Botrytis cinerea Causing Gray Mold Disease on Tomato Fruits

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Abstract: Gray mold disease caused by *Botrytis cinerea* is a damaging postharvest disease in tomato plants, and it is known to be a limiting factor in tomato production. This study aimed to evaluate antifungal activities of *Vernonia amygdalina* leaf extracts against *B. cinerea* and to screen the phytochemical compound in the crude extract that had the highest antifungal activity. In this study, crude extracts of hexane, dichloromethane, methanol, and water extracts with concentration levels at 100, 200, 300, 400, and 500 mg/mL were shown to significantly affect the inhibition of *B. cinerea*. Among the crude extracts, dichloromethane extract was shown to be the most potent in terms of antifungal activities. The SEM observation proved that the treatment altered the fungal morphology, which leads to fungal growth inhibition. For the in vivo bioassay, the fruits treated with dichloromethane extract at 400 and 500 mg/mL showed the lowest disease incidence with mild severity of infection. There were 23 chemical compounds identified in *V. amygdalina* dichloromethane extract using GCMS analysis. The top five major compounds were dominated by squalene (16.92%), phytol (15.05%), triacontane (11.31%), heptacosane (7.14%), and neophytadiene (6.28%). Some of these significant compounds possess high antifungal activities. This study proved that *V. amygdalina* from dichloromethane extract could be useful for inhibiting gray mold disease on tomato fruit and has potential as a natural antifungal agent.

Keywords: *Vernonia amygdalina*; antifungal activity; *Botrytis cinerea*; phytochemical; tomato; gray mold disease

1. Introduction

In Malaysia, approximately 96.30% of tomato production comes from the highlands, including Lojing, Kelantan, and Cameron Highland, Pahang [1]. The temperatures of these two locations range from 18 to 22 °C, with a relative humidity of 93–95%, which are optimal conditions for the development of fungal pathogen. *Botrytis cinerea* is a fungal pathogen of gray mold disease that can infect dicotyledonous plants, including tomato. Subjected to scientific and economic importance, *B. cinerea* was ranked as the second top plant pathogen in the world [2]. In 2015, Tijjani et al. [3]

observed tomato fruits with gray mold symptoms in postharvest storage at Cameron Highlands with 65% disease incidence, and this is the first report of gray mold disease on tomato fruits in Malaysia.

B. cinerea infection can develop in the field and can also cause postharvest decay or remain latent until storage. Spore germination of this pathogen grows vigorously in higher relative humidity and low temperature [4]. Thus, in cold storage, it leads to the development of gray mold symptoms, and this disease spreads rapidly among fruits in the same packaging. Chemical fungicides are the most commonly used to control gray mold on tomato fruits, but the repeated use of synthetic fungicide can develop fungal resistance and be harmful to consumer health. A few modes of action from a new fungicide provide adequate protection for fresh tomatoes [5]. However, the residue and toxicity concerns may limit their use.

Among the postharvest strategies in controlling plant diseases, natural products offer a promising treatment to reduce the disease incidence of postharvest diseases. Natural products contain advanced chemical novelty compared to chemically synthesized products, and for this reason, researchers try to discover new bioactive compounds in plants [6]. Plant extracts also contain beneficial secondary metabolites such as phenolics, tannins, coumarins, quinones, flavonoids, saponins, terpenoids, and alkaloids. These compounds have been proven to be potentially significant in plant protection as antimicrobial agents [7]. Many experiments have been conducted using plant extracts to control *B. cinerea* pathogen, which causes gray mold disease. Soylu et al. [8] found that essential oils extracted from rosemary and lavender could cause hyphae shriveled, protoplast leakage, conidia loss, and cytoplasmic coagulated on *B. cinerea* morphology. The extracted essential oil of fennel, cinnamon, and anis have also shown fungicidal effects on *B. cinerea* in in vitro and in vivo tests [9]. Moreover, the extraction of oregano and lemon effectively lowered the disease severity of gray mold disease in tomatoes, strawberries, and cucumbers [10]. In recent findings, stilbene extracted from grapevine leaves possessed antifungal activity of *B. cinerea* by inhibiting the mycelium growth and simultaneously reducing the necrotic lesion [11].

Bitter leaf is scientifically known as *Vernonia amygdalina*. In Africa and Asia, it is commonly used as a medicinal plant [12]. Various parts of *V. amygdalina,* including the leaf, root, and stem have been used for their antidiabetic, antioxidant, antimicrobial, anticancer, anti-inflammatory, and antiplasmodial effects [13]. Among the plant parts, researchers identified that the leaf part accumulates the highest chemical constituents and nutritional compositions [14]. Detailed investigations in the compound purification of *V. amygdalina* extract discovered many promising active compounds; for example, flavonoids, triterpenoids, saponins, tannins, sesquiterpene lactones, alkaloids, terpenes, phenolics, and steroidal glycosides [12]. According to Akowuah et al. [15], the extract from *V. amygdalina* was non-toxic in mice when exposed to up to 2000 mg/kg/day for 28 days.

To date, most researchers have focused on *V. amygdalina* crude extract in order to uncover its potential as an antifungal agent for the management of plant disease. Recent findings found that *V. amygdalina* ethanol extracts showed a good ability to inhibit postharvest fungal pathogens *Rhizopus stolonifer* and *Fusarium moniliforme* [16,17]. In another study, in an in vitro test using an ethanol crude extract of *V. amygdalina* at 300 mg/mL, the growth of *Cercosporella persica* and *Curvularia lunatus* were completely inhibited [18]. However, there has been no report on the antifungal activity of *V. amygdalina* extracted from different polarities of solvent against *B. cinerea* isolated from tomato. Therefore, this study aimed to (a) evaluate in vitro antifungal activities of *V. amygdalina* crude extract against *B. cinerea* in tomato; (b) study the effect of *V. amygdalina* extract on the morphology of *B. cinerea*; (c) determine in vivo antifungal activities of *V. amygdalina* crude extract against *B. cinerea*; and (d) screen the chemical constituents in *V. amygdalina* extract that are responsible for antifungal activities.

2. Materials and Methods

2.1. Plant Materials

Mature plants of *V. amygdalina* were collected from the local supplier in Puchong, Selangor and verified by a botanist from the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen (SK 3280/18) was deposited in the herbarium of the same institute. The plants were thoroughly cleaned, and the stems were cut into 25 cm lengths and planted in Ladang 15, Universiti Putra Malaysia (geographical coordinates: 2°59′19.8′′ North, 101°43′50′′ East, Malaysia) in December 2017. After two months, approximately 17 kg of matured leaves were harvested and brought to the Postharvest Laboratory, Universiti Putra Malaysia. The leaves were rinsed under running tap water to remove dirt, shade-dried for one week, and oven-dried at 40 °C for 4 h. The dried leaves were ground for two minutes using a high-speed grinder. The powdered sample was kept in an airtight container for further extraction processes.

2.2. Preparation of V. amygdalina Crude Extracts

The organic solvents in analytical grade (99% minimum purity) of hexane (Bendosen Laboratory Chemicals, Shah Alam, Malaysia), dichloromethane (DCM, Macron-Fine Chemicals, France), methanol (HmbG Chemicals, Hamburg, Germany), and acetone (Bendosen Laboratory Chemicals, Shah Alam, Malaysia) were used in the crude extraction process. The sequential extractions were performed according to the method as described by Haron et al. [19]. The sequential extraction procedure was shown in Figure 1. Each of the extracts was concentrated in a Buchi rotary evaporator until a sticky dark green crude extract was obtained. The highest percentage of crude extract yield was methanol (15.34%), followed by dichloromethane (DCM) (4.40%) and hexane (2.62% g). The crude extracts were kept in an airtight jar and stored at 4 °C.

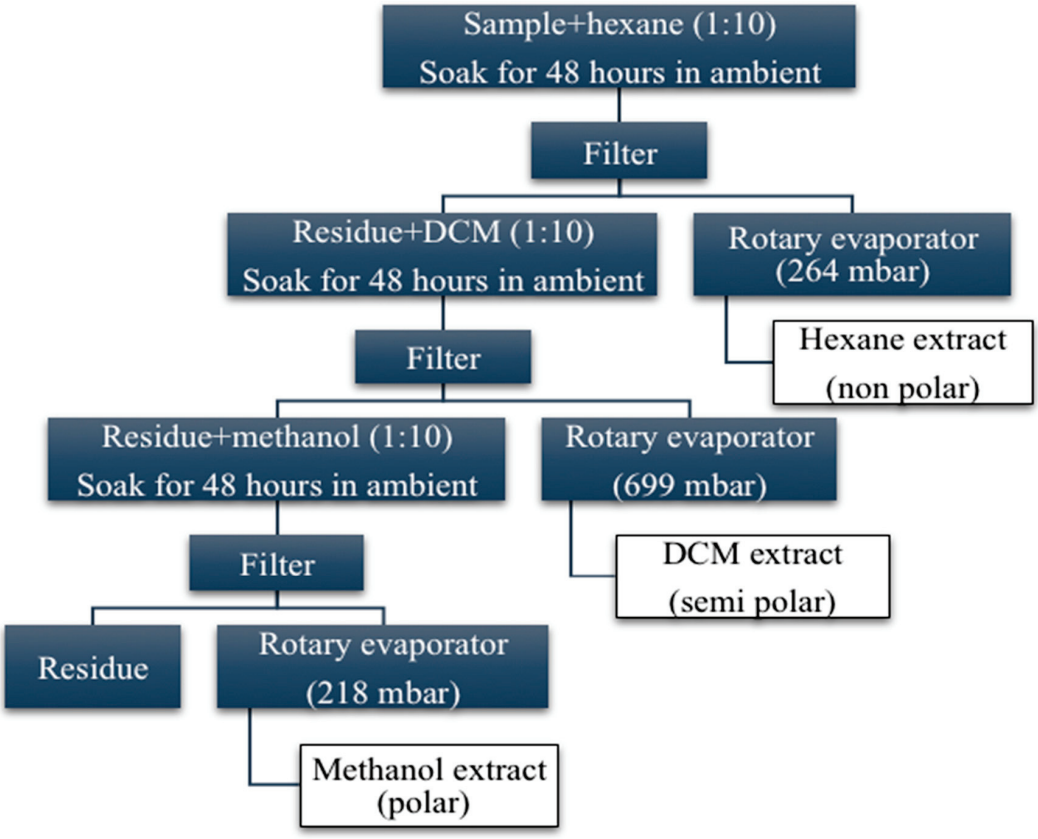

Figure 1. Sequential extraction procedure of *V. amygdalina.*

For the aqueous extract, 1 kg of the sample was soaked in 10 L of sterile distilled water and sonicated for 30 min at 50 °C. Then, it was filtered by using filter paper. The filtration was stored at –80 °C in a deep freezer (Ultra Low Temperature Freezer DW-86L386, Haier Medical and Laboratory Co., Ltd., Shandong, China). Next, it was freeze-dried in a freeze dryer (Christ Freeze Dryer BETA 1-8LD, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for three days. The obtained crude extract was ground using a pestle and mortar to obtain a 389.5 g fine powder and kept at 4 °C for further use.

2.3. Preparation of B. cinerea

B. cinerea was isolated from the diseased tomato fruits. The pure culture of the fungus was sub-cultured in potato dextrose agar (PDA) media. The identification of this specific fungus was viewed under a microscope for morphology characteristics. The morphological and molecular characteristics of *B. cinerea* were similar to the description of Javed et al. [20] and Tijjani et al. [3].

2.4. In vitro Evaluation for V. amygdalina Antifungal Activity

The crude extract was dissolved in 1 mL of acetone and sterilized using 0.4 μm syringe filter (Sartorius). Next, the extract solution was mixed with 15 mL of PDA in a sterile vial. The mixture was vortexed thoroughly and poured into an 85-mm Petri dish. The agar in Petri dishes was allowed to solidify to become poison agar. Poison agar medium was prepared individually at the concentration of 100, 200, 300, 400, and 500 mg/mL for each crude extract. Mycelial plugs from the pure culture of *B. cinerea* with 5-mm diameters were transferred to the center of the Petri dish containing PDA. Then, the plates were put into incubator (Model LM-575RD, Yihder Technology Co. LTD, Taipei, Taiwan) and incubated at 20 ± 2 °C for eight days to produce full-plate growth of mycelium with conidia. The edge of the active growing fungal plug (4 mm) from *B. cinerea* was taken and placed at the center of poison agar. The Petri dishes were incubated up to 8 days in an incubator (20 °C), and the fungal growth was observed. The percentage of radial growth (*PIRG*) was calculated after eight days of incubation as follows.

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

*R*1 and *R*2 are radial growth of fungus for control and extract, respectively.

2.5. Microscopic Observation Using a Scanning Electron Microscope (SEM)

The plates that contained the highest in vitro antifungal activities were viewed under SEM to confirm the fungal inhibition. The plugs (1 × 1 cm) were harvested from the control and DCM-treated plates, respectively. The sample preparation protocol followed that described by Heckman et al. [21]. Each plug was fixed in 2.5% glutaraldehyde for five hours at 4 °C. Next, the plugs were washed with 0.1 M sodium cacodylate buffer for three changes of 10 min each. One percent of osmium tetroxide was used in the post-fixed process for two hours at 4 °C. Then, the plugs were rewashed with 0.1 M sodium cacodylate buffer for three changes of 10 min each. A series of acetone was applied every 10-min interval in the dehydration process (35%, 50%, 75%, 95%) including 100% acetone for three changes every 15 min. The specimens were transferred into a specimen basket and put into a critical dryer for 1.5 h. After that, all specimens were stuck onto the stub and sputter-coated (Baltec SCD005) with gold in an ion sputter for two minutes. All sample specimens were viewed by microscope examination using JEOL JSM-6400 SEM.

2.6. Antifungal Activities of V. amygdalina by In Vivo Bioassay

The fresh and premium quality of tomato fruits in maturity index 3 (green with slight red) was selected. Two hundred of the selected fruits were washed under running tap water and air-dried for two hours. Then, the fruits were sprayed with 70% (*v*/*v*) ethanol and air-dried in laminar flow at ambient temperature for 30 min. Next, 120 fruits were dipped into the three most effective extract solutions

that showed the highest antifungal activity from in vitro bioassay (DCM at 300, 400, and 500 mg/mL) for five minutes. For negative control, 40 fruits were dipped in sterile distilled water and acetone. Meanwhile, another 40 fruits that were dipped in 0.5g/L Kenlate fungicide solution (active ingredient: 50% w/w benomyl) were used as a positive control. All treated fruits were excised using sterile cork borer (2 mm deep and 5 mm wide) at the equatorial side.

The preparation method of fungal plugs for in vivo bioassay was similar to that for the in vitro bioassay. The fresh fungal plug at 4 mm was harvested using a cork borer at the outermost layer of mycelium and inserted at the excised treated fruits. Finally, the fruits were kept in plastic boxes (40 fruits/box) and stored in ambient temperature at 95% relative humidity for five days [22]. Each treatment was replicated four times. Each replication consisted of ten fruits, and the experiment was repeated twice. At the end of storage duration, disease incidence and disease severity index (DSI) were determined using the following equations.

$$Disease\ incidence\ (\%) = \frac{Number\ of\ infected\ fruits}{Total\ number\ of\ fruit\ per\ treatment} \times 100$$

$$DSI = \sum \frac{a \times n}{AB} \times 100$$

where a = disease scale, n = number of fruits in a specific scale, A = highest disease scale, B = total number of fruits.

The disease severity was evaluated based on scale 0 to 4 as described by Rosero-Hernández et al. [23] using the following scale: 0 = no visible symptoms of fruits (no infection); 1 = 1–25% inoculated area covered with slight necrotic and water-soaked lesion (mild infection); 2 = 26–50% of the inoculated area covered with necrotic, white to gray mycelia and water-soaked lesion (moderate infection); 3 = 51–75% of fruits are necrotic with spore mass appeared and water-soaked (severe infection); 4 = >76% necrotic tissue appears soft, watery and decayed (very severe).

2.7. Screening for V. amygdalina Chemical Constituents Using Gas Chromatography-Mass Spectrometry (GCMS) Analysis

The crude samples at 0.02 g were dissolved in 1 mL acetone (HPLC grade, Fisher Chemical, USA). The solution was vortexed and filtered using a 0.02 µm filter syringe (Sartouris). The sterile crude extract was inserted into the HPLC vial and analyzed using GC-MS QP2010 Ultra (Shimadzu, Kyoto, Japan) comprising a gas chromatograph interfaced with a mass spectrometer. The compound separation was carried out using a Rxi-5MS fused silica capillary column of 30 m × 0.25 mm internal diameter (di) and 0.25 mm in film thickness (Restek GmbH, Homburg, Germany). The conditions for analysis were set as follows: column oven temperature was programmed column oven temperature at 50 °C, injection temperature maintained at 250 °C, injection mode split, flow control mode at the pressure of 37.1 kpa, total flow of 11.8 mL/min at 1 mL/min, column flow of 0.8 mL/min, split ratio of 10.0, ion source temperature 200 °C, interface temperature 250 °C, solvent cut time 2.0 min, and detector gain mode by relative and detector gain 0.88 kV + 0.00 kV. The column oven temperature was set at 50 °C (maintained for 3 min), raised at 10 °C/min to 280 °C (maintained for 3 min), and finally maintained at 300 °C for 10 min. Mass spectra were taken at the start time of 2.5 min and end time of 93.0 min. The ACQ mode Scan was carried out at an event time of 0.10 s, with a scan speed of 10,000 m/s. The start m/z was 40, and the end m/z was 700. Constituents were identified based on data libraries by analyzing and comparing mass spectra (FFNSC1.3.lib, WILEY229.lib and NIST11s.lib).

2.8. Experimental Design and Statistical Analysis

For the in vitro bioassay, the experiment was conducted in completely randomized design (CRD) with four replications. The test was arranged in two factorial analysis consisting of 4 types of crude extracts (hexane, DCM, methanol, aqueous) × 5 concentration levels (100, 200, 300, 400, and 500 mg/mL).

For the in vivo bioassay, the experimental design was also in CRD and four replications, thus leading to a total of 80 experiments. The data were analyzed using analysis of variance (ANOVA), and the means were separated using the least significant difference (LSD) test at $p \leq 0.05$. The data analysis was carried out in SAS software (version 9.4).

3. Results

3.1. In Vitro Antifungal Activities of V. amygdalina Crude Extract against B. cinerea

In this study, PIRG was measured and calculated to determine the antifungal activity of four types of crude extracts at five different concentrations. As shown in Table 1, both the main factors of the crude extracts and the concentration levels significantly inhibited the in vitro growth of *B. cinerea*. There was also a highly significant interaction effect between crude extract and concentration levels (CE × CL) on *B. cinerea* growth.

Table 1. Main and interaction effects of *V. amygdalina* crude extracts and concentration level on PIRG of *B. cinerea*. PIRG: percentage of radial growth.

Factors	PIRG (%)
Crude extracts (CE)	
Hexane	22.65 ± 2.34d [x]
Dichloromethane	64.94 ± 2.19a
Methanol	45.15 ± 2.28b
Aqueous	25.18 ± 2.32c
Concentration levels (mg/mL) (CL)	
100	25.07 ± 4.47d
200	34.04 ± 4.93c
300	40.04 ± 4.25b
400	48.05 ± 4.61a
500	50.18 ± 4.29a
Significance	
CE × CL	**

Values are expressed as mean ± SD. [x] Means with the same letters within a column and each factor are not significantly different at $p \leq 0.05$ using the LSD test. ** $p \leq 0.01$.

The results indicate that the DCM crude extract of *V. amygdalina* possessed the most potent antifungal activity, exhibiting a fungistatic effect on the growth of *B. cinerea* followed by methanol, aqueous, and hexane crude extract (Figure 2). As expected, higher concentrations of 400 and 500 mg/mL showed the highest PIRG of *B. cinerea* for all crude extracts. However, both concentration levels showed no significant differences in *B. cinerea* inhibition growth. The effects of PIRG of *B. cinerea* between methanol crude extract at 200 and 300 mg/mL as well as hexane crude extract at 100 and 200 mg/mL were not significant. Within the concentration level of DCM extract, the PIRG increased as the concentration level was raised to 400 mg/mL. This result shows that the maximum inhibitory effects of *B. cinerea* radial growth were DCM at 400 and 500 mg/mL, with 74.85% and 75.7% inhibition, respectively.

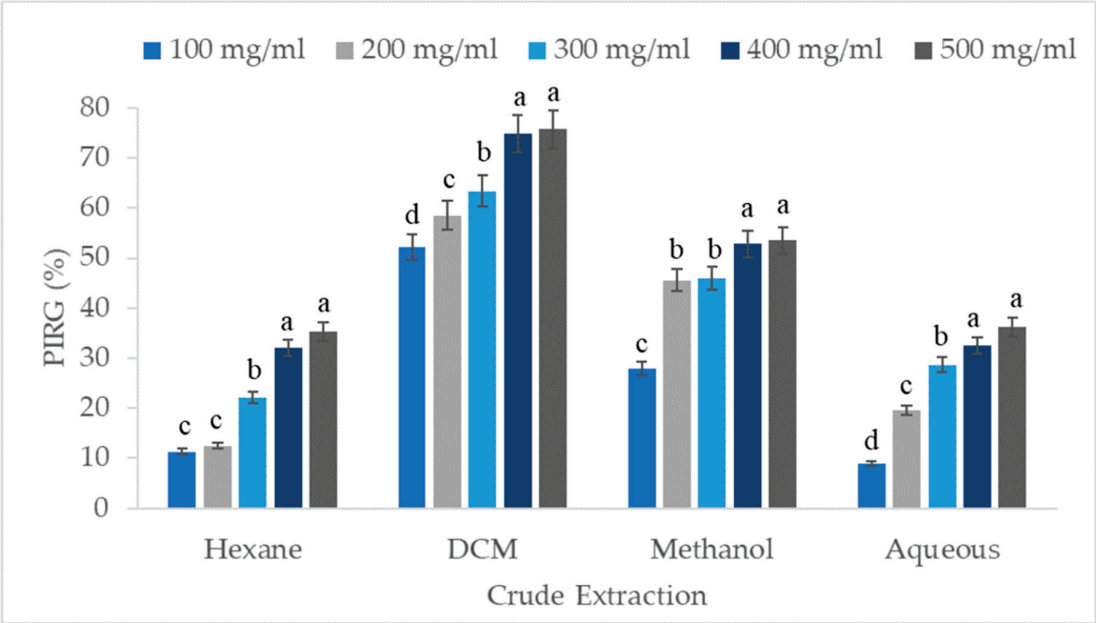

Figure 2. Effect of crude extraction of *V. amygdalina* at various concentrations on the PIRG of *B. cinerea* after eight days of incubation. Means with the same letter within each crude extraction are not significantly different at $p \leq 0.05$ using the least significant difference (LSD) test.

3.2. Effect of V. amygdalina Crude Extract on the Morphology of B. cinerea

The morphology of *B. cinerea* was altered after being exposed to *V. amygdalina* DCM treatment. Under SEM observation, the mycelia of this fungus were shriveled, retarded, and agglutinated while the conidia underwent shrinkage (Figure 3C–F) compared to the control, which had a slender shape, and was massive, and for which the conidiophore was grape-shaped with ellipsoidal conidia (Figure 3A,B).

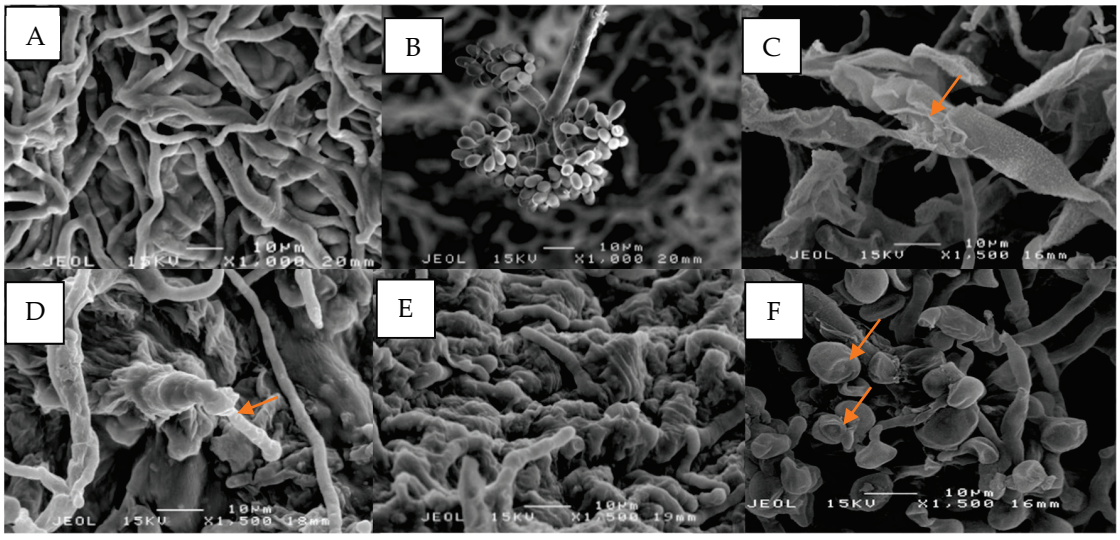

Figure 3. Effects of dichloromethane (DCM) crude extract on *B. cinerea* at 400 and 500 mg/mL on mycelium morphology viewed under SEM. (**A**) Healthy mycelium are slender and uniform, with a smooth surface and an intact structure in the control plate; (**B**) Healthy conidiophore from the control plate; (**C**) Mycelia were ruptured, folded with edge burrs, and sheet-like structure at 400 mg/mL; (**D**) The hyphae tip was wrinkled and deformed at 400 mg/mL; (**E**) Agglutinated mycelia at 500 mg/mL; (**F**) The conidia were shrunken at 400 mg/mL.

3.3. In Vivo Antifungal Activities of V. amygdalina Crude Extract against B. cinerea

The in vivo experiment showed that all fruits from the negative control were infected with gray mold disease with 100% of disease incidence (Table 2). Meanwhile, the artificially inoculated tomato fruits that were dipped in *V. amygdalina* DCM treatment at 400 and 500 mg/mL had reduced disease incidence of gray mold by 50% and 53.13% compared to the control fruits, respectively. However, the effect on the disease incidence at both concentrations was not significantly different.

Table 2. Percentage of *B. cinerea* incidence on tomato treated fruits.

Treatment	Disease Incidence (%)
Negative control	100 ± 0.00a ^{X}
Benomyl	68.75 ± 2.69b
DCM 300 mg/mL	60.41 ± 2.69c
DCM 400 mg/mL	50.0 ± 3.40d
DCM 500 mg/mL	46.88 ± 3.13d

Values are expressed as mean ± SD. ^{X} Means with the same letters are not significantly different at $p \leq 0.05$ using the LSD test.

Artificially inoculated tomato that was treated with *V. amygdalina* DCM extract showed significantly lower disease incidence compared to the commercial fungicide (benomyl) treatment. Regarding the DCM treatments, DCM at 400 mg/mL could reduce incidence by 2.23% compared with DCM at 300 mg/mL.

Among the infected fruits, the disease severity displayed a different pattern according to the treatment. Table 3 indicated that the fruits in negative control were observed 27.28% in disease severity index, with a value of 2 for severity scale and moderate infection category. The necrotic tissue in control fruits was covered with white to gray mycelia and water-soaked lesions.

Table 3. Percentage of disease severity index on tomato treated fruits.

Treatment	DSI (%)
Negative control	27.28 ± 0.29a ^{X}
Benomyl	10.84 ± 0.69b
DCM 300 mg/mL	4.50 ± 0.53c
DCM 400 mg/mL	2.27 ± 0.12d
DCM 500 mg/mL	2.19 ± 0.05d

Values are expressed as mean ± SD. ^{X} Means with the same letters are not significantly different at $p \leq 0.05$ using the LSD test.

The tomato fruits that were treated with *V. amygdalina* DCM extract at concentration 300–500 mg/mL with severity scale 1 were shown to have a significantly lower percentage of disease severity compared to chemical fungicide (benomyl). Thus, the treatment of *V. amygdalina* DCM extract could be an alternative fungicide, constituting a natural antifungal agent against the gray mold disease of tomato fruits. However, nonetheless, the gray mold disease severity of all treated fruits of DCM extract treatments and benomyl constituted a mild infection.

3.4. Phytochemical Screening of DCM Crude Extract

GCMS analyses of the crude extract led to the identification of 23 chemical constituents in the DCM crude extract of *V. amygdalina* (Figure 4).

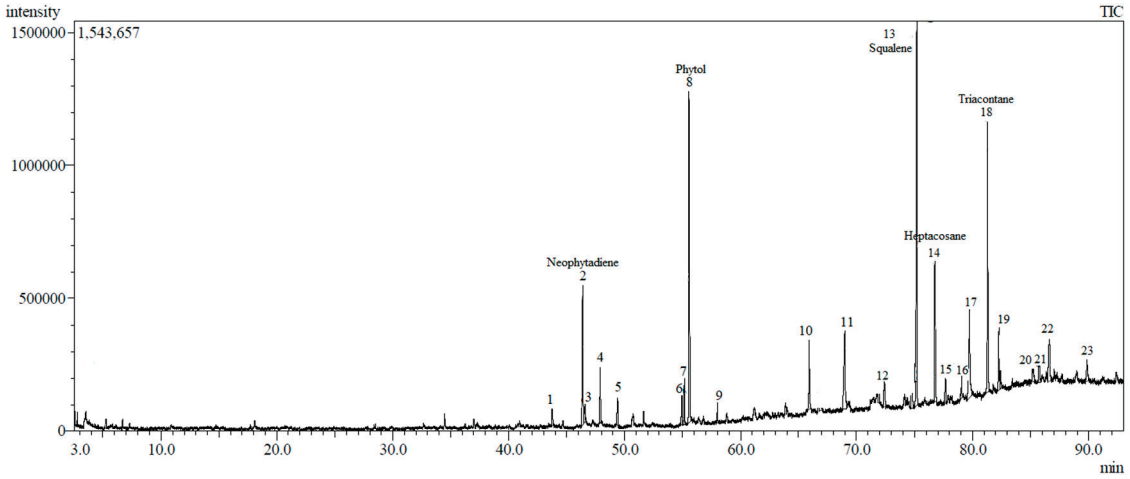

Figure 4. Ion chromatogram of DCM crude extract using GCMS.

The identified compounds are arranged according to their elution order on silica capillary columns. The extract contains a complex mixture consisting of mainly triterpenoid, diterpene alcohol, sesquiterpene, and hydrocarbon lipid. The top five major compounds are squalene (16.92%), phytol (15.05%), triacontane (11.31%), heptacosane (7.14%), and neophytadiene (6.28%) (Table 4).

Table 4. Chemical composition in DCM crude extract of *V. amygdalina.*

Peak	Retention Index **	Compound Name	Chemical Group	Area (%)
1	1763	Loliolide	Monoterpenoid hydroxylactones	0.76
2	1839	Neophytadiene	Sesquiterpene	6.28
3	1841	Phytone	Terpene ketone	0.90
4	1860	2-Hexadecen-1-ol	Acyclic diterpene	0.79
5	1882	3,7,11,15-tetramethyl-2-hexadecen-1-ol	Acyclic diterpene	1.46
6	1966	Hexadecanoic acid	Fatty acid	1.06
7	2089	9,12-Octadecadienoic acid	Fatty acid	1.19
8	2119	Phytol	Diterpene alcohol	15.05
9	2147	Linolenic acid	Fatty acid	1.86
10	2499	l-caryophyllene	Bicyclic sesquiterpene	3.35
11	2577	γ-Elemene	Sesquiterpene	5.72
12	2711	1,3,7-Nonatriene-1	Monoterpene	1.35
13	2830	Squalene	Triterpene	16.92
14	2892	Heptacosane	Hydrocarbon lipid	7.14
15	2577	Geranyl linalool	Monoterpenoid	1.12
16	2993	Tetratriacontane	Hydrocarbon lipid	1.02
17	3021	Unknown	-	6.41
18	3097	Triacontane	Hydrocarbon lipid	11.31
19	3139	α-Tocopherol	Vitamin E	6.04
20	3272	Stigmasterol	Stigmastane	0.98
21	3290	α-spinasterol acetate	Stigmastane	1.37
22	3295	Chondrillasterol	Triterpene (sterol)	2.90
23	3472	α-tocopherol acetate	Vitamin E	1.46

** Retention index on the Rxi-5MS silica capillary column.

The other characteristic constituents of the crude extract are loliolide, phytone, 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, hexadecanoic acid, 9,12-octadecadienoic acid, linolenic acid, caryophyllene, γ-elemene, 1,3,7-nonatriene-1, geranyl linalool, tetratriacontane, α-tocopherol, stigmasterol, α-spinasterol acetate, chondrillasterol, α-tocopherol acetate, and one unknown compound were found to be minor components of DCM *V. amygdalina* leaves extract in the present study. Chemical residue of solvents used in the extraction was not found by GCMS analysis.

4. Discussion

Extraction methods involve the separation of the active compound of plant tissues from inactive components using selective solvents. During extraction, solutions defuse into solid plant material and solubilize compounds with similar polarity. The present experiments showed that extracts obtained from *V. amygdalina* contain essential components for the inhibition of mycelial growth of *B. cinerea* pathogenic on tomato plants. However, each of the crude extracts was varied in terms of their antifungal activities. Among the crude extract, DCM extract (semi-polar) of *V. amygdalina* showed the most potent inhibition on *B. cinerea* growth. As reported in other cases [24], DCM extract fraction of *Pseudognaphalium robustum* reduced the in vitro mycelial growth of *B. cinerea* at 45.5 μg/mL by 50%. They stated that it also affected the conidial germination of the *B. cinerea* by reducing oxygen consumption and interrupting plasma membrane integrity.

In contrast, Righini et al. [25] found that the water extract of *Anabaena* sp., *Ecklonia* sp., and *Jania* sp. inhibited the in vitro growth of *B. cinerea* at 2.5, 5.0, and 10.0 mg/mL. Generally, it appears that the inhibitory effect of the plant extracts varies depending on the specific plant and solvent used with no specific trend related to the polarity of the solvent. The usage of different solvent systems will extract a diversity of molecules with distinct polarities. For instance, methanol tends to extract a diversity of compound groups such as polyphenols, glycosides, and flavonoids [26], which can contribute to the fungal inhibitory effect of the extract. DCM and hexane tend to extract mainly semi-polar and nonpolar constituents such as terpenoids, fats, and fatty acid [27]. It would appear that both polar and nonpolar constituents contributed to the antifungal activity of the plant extracts.

The percentage of fungal inhibition in amended PDA medium was also dependent on concentration, and the most significant reduction in mycelial growth was obtained with the highest concentration of crude extract. A similar result was observed by other researchers using ethanol leaf extract of *V. amygdalina* against tomato diseases [16]. This could be due to the level of composition antifungal compounds obtained from the crude extract. The inhibitory effect of DCM extract increased as the concentration increased, showing more than 50% growth inhibition at all concentrations. However, the inhibition growth of *B. cinerea* showed no significant difference in any crude extract at concentrations of 400 or 500 mg/mL. A possible reason for this circumstance is the low water solubility of the antifungal compounds, which limits the miscibility in the agar medium through the poison agar method. Meanwhile, the hydrocarbon components either remained on the medium surface or evaporated, depending on its nature. According to Krzyśko-Łupicka et al. [28], the biocidal action from the plant extract also depends on the chemical composition, concentration, and phytopathogenic fungi strains.

In vivo experimental results show that negative control fruits were 100% infected with gray mold disease with a severity value of 2. The necrotic tissue in negative control fruits was water-soaked and covered with fungal mycelia. The highest *Botrytis* incidence was observed in tomatoes six days after inoculation [29], and in kiwi seven days post-inoculation [30]. Meanwhile, the artificially inoculated tomato fruits that were dipped with the two highest concentrations of DCM extract resulted in the highest reduction in disease incidence of gray mold. The possible reason for these results is the concentration level of antifungal compounds from plant extract being sufficient to control the inoculum of *B. cinerea* on tomato fruits. This statement is supported by the disease severity of gray mold on tomato in the present study, which only causes a mild infection on the fruits. The results provide baseline information for the potential use of the crude extract in the treatment of postharvest gray mold disease. This was necessary because this disease was a latent field infection [31]. However, the treatment data we obtained did not prevent the onset of gray mold disease because the percentage of disease incidence reduction was not 100%, although it was generally low. Fillinger and Elad [32] suggested that *B. cinerea* is very challenging to control due to its broad host range, different mode infection, and both asexual and sexual stages enabling it to survive in favorable or unfavorable conditions.

Meanwhile, it is well known that the fungicide activity of benomyl on a broad spectrum of phytopathogenic fungi was due to its ability to be absorbed by the phytopathogen cells. However, the present study found that benomyl treatment on *B. cinerea* has a fungistatic activity. Benomyl has

been applied as a systemic fungicide since 1970 and was used to control *B. cinerea* in 1971 [33]. Methyl 2-benzimidazole carbamate (MBC) is the major metabolite of benomyl and is primarily responsible for the fungitoxicity. Hammerschlag and Sisler [34] indicated that the primary metabolites were inhibited by the synthesis of DNA, interrupted fungal cell division, and inhibited cytokinesis. However, in this case, the benomyl treatment failed to act as a fungicidal. This could be due to the fungal resistance to this fungicide, since benomyl has been used for many years.

In the present study, the highest antifungal activity of DCM extract against *B. cinerea* could be associated with the presence of 23 bioactive compounds identified using GCMS. The major compounds were dominated by squalene (16.92%), phytol (15.05%), triacontane (11.31%), heptacosane (7.14%), and neophytadiene (6.28%). Squalene is the most abundant in this crude extract. It is a naturally occurring triterpenoid and a precursor for the synthesis of secondary metabolites such as sterols, hormones, or vitamins. Squalene has been shown to have excellent antioxidant, anticancer, antibacterial, and antifungal biological activities [35]. In pharmacognosy, squalene is extensively used as an excipient for disease management and therapy [36]. Gnamusch et al. [37] found that squalene at high concentration resulted in disturbances in the fungus cellular membranes and interfered with essential membrane functions. The non-toxic chemical nature of lipids makes them excellent carriers as well as their ability to permeate the cell membrane of fungus due to their lipidic nature.

Phytol is the second-highest compound in this extract. It was classified in the diterpene group. Haque and co-workers [38] explained that terpenoids from the plant extract reduce the mitochondrial content of fungus, which could alter the ATP generation and level of reactive oxygen species. Consequently, the mitochondria of the fungus become dysfunctional. Yoshihiro et al. [39] reported that diterpene of phytol could disrupt the cell membranes of the fungus, resulting in K+ ions leaking from the cells, and causing the fungus hyphae to wither.

The aliphatic hydrocarbons of triacontane (n-C30) and heptacosane (n-C27) are identified as major components in this extract. Both long-chain alkanes were found on the surface of plant parts and in abundance in the epicuticular wax of matured leaves [40]. They acted as a physical barrier on the plant in terms of water loss, irradiation, phytopathogen attack, and insect herbivores. The appearance of the epidermal wax of the *V. amygdalina* leaf surface was proven by Eltahir and AbuEReish [41]. They reported that under SEM observation, the epidermal wax is present in large quantities at the abaxial leaf part compared to the adaxial part. Yin et al. [42] found that the hydrocarbon waxes inhibited *Alternaria* rot of pear by stunting the spore germination and mycelial growth of *Alternaria alternata*. However, to date, the specific mechanisms of action of these hydrocarbon compounds as antifungal agents against phytopathogenic fungi have been less reported.

Neophytadiene is a sesquiterpene compound and was found to be a significant component in DCM extract. Neophytadiene is an active compound with antibacterial, antifungal, antipyretic, and antioxidant activities [43]. This sesquiterpene compound could pass through the cell wall, interrupt the cell membrane function, and destroy the fungal mitochondria structure [44]. In a previous study, the sesquiterpene compound isolated from *Magnolia grandiflora* was also proven to have fungicidal effects against *A. alternata* and *F. culmorium* [45]. Neophytadiene extracted from *Daucus carota* subsp. *sativus* was identified as a major compound and showed protective as well as preventive activity against *B. cinerea* in strawberry [46].

On the other hand, some of the chemical constituents that appear in lower amounts in this extract, such as terpenoid, steroid, and fatty acid, might also contribute to the antifungal activity. Howard et al. [47] stated that *V. amygdalina* contains bioactive sesquiterpene lactones that possess highly antifungal effects. Similarly, Ivanescu et al. [48] explained that the mechanism of biological activity of alkylating sesquiterpene lactones and the nucleophile sulfhydryl group in proteins led to the disruption of cell function that caused cell wall damage of the fungus. It is possible that the minor chemical constituents might also be related to synergism effects with major compounds, inhibiting *B. cinerea* growth.

The mechanism action of antifungal compounds of the *V. amygdalina* extract on the fungal inhibition was observed under SEM. The hyphae of *B. cinerea* exposed to the phytochemical compounds of *V. amygdalina* extract revealed alterations in the hyphal morphology. The mycelia became twisted and folded with a jagged edge. Some mycelia were agglutinated, with withered hyphae tips. Another important observation was the shrinkage of conidia after the treatment. This could prevent the dispersion of the gray mold disease of fruits to the adjacent fruits, since the asexual spores of *B. cinerea* are abundant and easily dispersed by wind or water. The morphology alteration of fungus was related to the secondary metabolites from the plant extract that acted as antifungal substances to restrict the fungal growth [49]. This mode of action was called antibiosis. The antibiosis happened when the secondary metabolites from the plant extract inhibited or restricted the growth of the pathogen. The secondary metabolites inhibited fungal growth through cell membrane disruption, cell wall synthesis inhibition, mitochondrial dysfunction, cell division inhibition, protein synthesis inhibition, and efflux pump inhibition [32]. In this case, the composition of major antifungal compounds, including squalene, phytol and neophytadiene, as well as minor antifungal compounds, acted as synergistic effects in controlling the *B. cinerea* development.

5. Conclusions

This study provides evidence that *V. amygdalina* extracts of DCM at 400 and 500 mg/mL had the highest antifungal activities against *B. cinerea* through in vitro and in vivo bioassays. The treatment altered the fungal morphology and inhibited fungal growth. The chemical constituents in this plant extract have the potential to be a natural antifungal agent. Thus, we propose an alternative disease management strategy using *V. amygdalina* extract to control gray mold disease on tomato. However, further research is necessary to elucidate the mechanism of action and develop the formulation to improve its efficacy and stability for use in postharvest disease control. The postharvest quality study should also involve a formulation application to make sure the quality of fresh fruits is optimal and that the fruits are safe to consume.

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The Phytochemical, Antifungal, and First Report of the Antiviral Properties of Egyptian *Haplophyllum tuberculatum* Extract

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Abstract: In this study, ethanol whole plant extract (WPE) of *Haplophyllum tuberculatum* was characterized and tested for its antifungal and antiviral activities against *Fusarium culmorum*, *Rhizoctonia solani* and tobacco mosaic virus (TMV). High Performance Liquid Chromatography (HPLC) analysis showed that the main phytochemical constituents of *H. tuberculatum* WPE were resveratrol (5178.58 mg/kg), kaempferol (1735.23 mg/kg), myricetin (561.18 mg/kg), rutin (487.04 mg/kg), quercetin (401.04 mg/kg), and rosmarinic acid (387.33 mg/kg). By increasing *H. tuberculatum* WPE at concentrations of 1%, 2%, and 3%, all of the fungal isolates were suppressed compared to the two positive and negative controls. Under greenhouse conditions, WPE-treated *Chenopodium amaranticolor* plants strongly inhibited TMV infection and significantly reduced TMV accumulation levels when compared to non-treated plants. Moreover, the induction of systemic resistance with significant increases in the transcriptional levels of the pathogenesis-related protein-1 (*PR*-1), chalcone synthase (*CHS*), and hydroxycinnamoyl-CoA quinate transferase (*HQT*) genes for treated plants were noticed at 3 and 5 days post-inoculation (dpi) for both assays. To the best of our knowledge, this is the first reported observation of the antiviral activity of *H. tuberculatum* extract against plant viral infections. Finally, the results obtained suggest that *H. tuberculatum* WPE can be considered a promising source of both antifungal and antiviral substances for practical use and for developing plant-derived compounds for the effective management of plant diseases.

Keywords: *Haplophyllum tuberculatum*; phytochemical analysis; HPLC analysis; antifungal property; tobacco mosaic virus; antiviral activity

1. Introduction

Haplophyllum is a genus belonging to the Rutaceae family. It is distributed in different floristic regions. The plant is rich in alkaloids, fixed oils, volatile oils, furanocoumarins, and several classes of compounds such as alkaloids, lignans, coumarins, and flavonoids have been isolated from the aerial parts of *Haplophyllum tuberculatum* [1]. Globally, plant viral diseases constitute severe threats to sustainable development and modern agriculture [2]. Among these viruses, the tobacco mosaic virus (TMV) is one of the most common viral diseases that causes economic losses of and severe damage to

quality and crop production worldwide [3,4]. Besides ranking in the top 10 plant viruses in molecular plant pathology, TMV is used as a model virus, and *Chenopodium amaranticolor* as a TMV-local lesion host for different biological studies [5]. Insect-borne viruses such as TMV and cucumber mosaic virus (CMV) can be controlled well by applying induced resistance (IR), even by a biological or chemical inducer [6,7]. In addition, in Egypt, the production of tomato faces the problem of insect-borne viruses, which are very difficult to manage because of their wide host range [8]. Systemic acquired resistance (SAR) is an inducible defense mechanism that plays a central role in disease resistance [9]. Moreover, the chemical induction of SAR treatment induces both pathogenesis-related (PR) protein accumulation and resistance to viruses, bacteria, and the fungus in Arabidopsis plants [10].

Recently, it has been demonstrated that the chemical induction of SAR treatment of vegetable plants protects them against root rot diseases [11]. Several biotic stresses can affect tomato plants more so than other vegetables. Moreover, they are capable of remaining in soil and plant residues for an extended period of time [12]. The soil-borne pathogenic fungus "biotic stresses" can lead to a decrease in crop production of the *Rhizoctonia solani* plant, which causes several injures in the bean of tomato crops [13]. *Fusarium* spp. are an abundant saprophyte in soil and organic matter and are found worldwide. Some strains cause vascular wilt disease in plants, including vegetables, bananas, and date palms such as *Fusarium culmorum*.

Most fungal species, such as the *Fusarium*, *Rhizoctoni*a, and *Penicillium* species, cause mold and discolor wood-based products [14,15]. However, chemical fungicides can induce further problems, harming other living organisms by reduction of useful soil microorganisms [16]. Therefore, alternative methods of pest control could be an approach to reduce the use of pesticides. For instance, biological control strategies are slowly replacing harmful pesticides due to the acceleration of developed biological control products and commercialized forms [17]. Recently, there has been a trend to develop an environmentally safe, long-lasting chemical fungicide based on plant metabolites as an alternative for the control of *Fusarium* diseases, benomyl, and captafol [18,19]. Many plants exhibit antifungal activities that could produce a variety of secondary metabolites against phytopathogenic fungi [20–22]. Plant-derived natural products and bioactive compounds include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, and coumarins, which are well-known examples for biofungicides [23–26].

The mycelial radial growth of *Fusarium oxysporum* is reduced by *Azadirachta indica*, *Calotropis procera*, *Citrullus cololcynthis*, *Datura stramonium*, and *Nicotiana tabacum* extracts [27]. Furthermore, *Cinnamomum burmanni* leaf aqueous extract efficiently suppresses the biomass and spore formation of *F. oxysporum f.* sp. *lycopersici* [28]. Meanwhile, the ethanol extracts from *Lowsonia inermis* and *Psidium guajava* are effective in inhibiting the *Fusarium* pathogen [29].

Most approaches applied to control TMV include treating plants with chemical pesticides or using breeding and transgenic plants. However, chemically synthetic pesticides harm the environment and human health; also, transgenic plants have not yet been universally accepted [30]. Therefore, there is still a high demand for discovering more alternative, environmentally friendly, and effective antiviral methods.

Plants are rich sources of bioactive constituents with an antiviral activity that can develop environmentally friendly methods of disease management [31]. Reports of the antiviral activities of plant crude extracts and their constituents against plant viral infection have increased during the last decade [32]. Many plant extracts of *Boerhaavia diffusa*, *Clerodendrum aculeatum*, *Mirabilis jalapa*, *Potentilla arguta*, *Sambucus racemosa*, and *Thuja orientalis* exhibit inhibitory effects against plant viruses [33–37]. Additionally, several virus-inhibiting compounds, including flavonoids, triterpenoids, alkaloids, and proteins, have been isolated from higher plants [31].

H. tuberculatum extracts have been noticed to exhibit insecticidal [38], nematicidal [39], antifungal, and antibacterial properties [40,41]. The polyphenolic and alkaloid compounds in the ethyl acetate extract from the leaves of *H. tuberculatum* may be significant contributors to the antioxidant activity of these extracts [42]. Leaf oil extracted from *H. tuberculatum* shows strong anticandidal activity against

Candida krusei at 30 μg/mL [43]. Also, essential oil has been found to inhibit the growth of *Curvularia lunata* and *F. oxysporum* [40].

The present study aimed to analyze the protective activity and inactivity of the ethanol extract of *H. tuberculatum* against the TMV for the first time. Additionally, the changes in the transcriptional levels of some defense-related genes and TMV accumulation levels at different time intervals were evaluated. Moreover, the antifungal properties of *H. tuberculatum* were assessed against two molecularly identified fungal isolates, namely, *F. culmorum* and *R. solani*.

2. Materials and Methods

2.1. Preparation of the H. tuberculatum Extract and HPLC Analysis of Phenolic Compounds

Whole plants (WP) of *H. tuberculatum* collected from the northwest of Egypt in April 2018 were air-dried at room temperature for one week. The dried WP was ground to a fine powder using a small laboratory mill. Approximately 100 g of the powdered WP of *H. tuberculatum* was extracted by the soaking method [44] with 200 mL of ethanol solvent for three days. After the extraction process was finished, the extract was filtered through a cotton plug and then with Whatman No. 1 filter paper. The filtered extract was concentrated by evaporating the ethanol solvent to obtain the *H. tuberculatum* whole plant extract (WPE). To prepare the concentration of the extract, the *H. tuberculatum* WPE was dissolved in dimethyl sulfoxide (10% DMSO = 10 mL DMSO (99.999%) + 90 mL distilled water), and the concentrations levels of 1%, 2%, and 3% were obtained. Then, 1, 2, and 3 g from the extract were dissolved in 100 mL of 10% DMSO to obtain the extract concentrations of 1%, 2%, and 3%, respectively. The extract was analyzed for its polyphenolic compounds using Agilent 1260 Infinity HPLC Series (Agilent, Santa Clara, CA, USA), equipped with a Quaternary pump and a Zorbax Eclipse Plus C18 column (100 × 4.6 mm i.d.) in *H. tuberculatum* WPE [15,44–48]. The analysis was carried out based on the 23 standard phenolic compounds [47].

2.2. Antifungal Property of Wood Treated with H. tuberculatum WPE

F. culmorum and *R. solani* as common molds were used for the antifungal bioassay previously isolated from twigs, trunks, and roots were collected from sweet orange trees showing cankers, dry root rot, wilt and decline at Bader district, Egypt. The isolated fungal colonies were characterized morphologically and molecularly by the internal transcribed spacer region of the rDNA (ITS) gene and the amplified fragments were sequenced and the generated sequences were deposited in Genbank under accession numbers MH352452 and MH352450, respectively [49,50]. Briefly, *Melia azedarach* wood samples were air-dried and prepared with an approximate dimension of 0.5 × 1 × 1 cm. The prepared wood samples were autoclaved for 20 min at 121 °C, and then left to cool. Three wood samples were used for each concentration for each fungus, as well as for the positive (25 μg of fluconazole) and negative (10% DMSO) controls. The antifungal activity of the wood-treated extract in terms of the inhibition percentage of fungal linear growth (IPFLG) was measured following our previous work [15,49–51], using the following formula; IPFLG (%) = $[(G_C - G_T)/G_C] \times 100$, where G_C and G_T represent the average diameters of the fungal colony of control and treatment, respectively.

2.3. Source of Virus, Inoculum Preparation and Antiviral Activity Assays

Egyptian TMV strain KH1 (Acc# MG264131) was propagated in *N. tabacum* and purified as previously described [52]. Then, 50 μL of 20 μg/mL TMV, diluted with 0.1 M phosphate buffer, pH 7.2, was used as the viral inoculum. The prepared concentration of the WPE of *H. tuberculatum* (200 μg/mL) diluted with sterile distilled H_2O was prepared from a stock solution of 2% of *H. tuberculatum* WPE dissolved in DMSO. A mixture of equal volumes of DMSO and sterile distilled H_2O was used as the negative control. By using the half-leaf method [47,53], with *C. amaranticolor* as a TMV-local lesion host, the assessment of the antiviral activity of WPE was evaluated according to the inhibition percentage toward number of local lesions. The inhibitory effects were calculated according to the following

formula: [I = (1 − T/C) × 100], where I is the inhibition effect, T is the number of local lesions on the treated halves of the leaves, and C is the number of local lesions on the non-treated halves of the leaves.

2.4. Protective and Inactivity of WPE Assays

Under greenhouse-controlled conditions, *C. amaranticolor* seeds were surface sterilized and sown in plastic pots (20 cm in diameter) filled with sterilized soil. At the 5–6th leaf stage, plants were subjected to two assays, and each assay had three treatments replicated three times.

In the protective assay, the upper-right halves of the leaves were treated with *H. tuberculatum* WPE 24 h before mechanical viral inoculation. In contrast, the upper-left halves of the leaves were inoculated with TMV only without any treatment [54,55]. Mock leaves treated with a mixture of equal volumes of DMSO, sterile distilled H_2O, and phosphate buffer with carborundum were used as the controls. The local lesion development numbers were recorded at 3–5 days post-inoculation (dpi).

In the inactivity assay, the upper-right halves of the leaves were mechanically inoculated with an *H. tuberculatum* WPE–TMV mixture, in which an equal volume of *H. tuberculatum* WPE was mixed with the same amount of purified TMV and incubated for 1 h. In contrast, the upper-left halves of the leaves were mechanically inoculated with TMV only without any treatment. The observed number of local lesions was recorded 4–5 dpi.

2.5. Plant Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the *C. amaranticolor* halve of the leaves (0.1 g fresh weight), which were collected at 3 and 5 dpi using the RNeasy plant mini kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). After treatment with RNase-free DNase to eliminate genomic DNA, the concentration and quality of the extracted RNA were determined at *A*260/*A*280 and *A*260/*A*230 using SPECTROstar Nano (BMG Labtech, Ortenberg, Germany). In contrast, the integrity of the RNA was assessed by the agarose gel electrophoresis technique [56]. First-strand cDNA was synthesized using 1 μg of total RNA with random oligohexamers and oligo (dT) primers, as described previously [57]. Then, RT-PCR was performed in two steps: 42 °C for 1 h and then 72 °C for 10 min. The reaction mixture was stored at −20 °C until used.

2.6. Quantitative Real-Time PCR (qPCR) Assay and Data Analysis

The effects of *H. tuberculatum* WPE on the expression of the accumulation levels of the TMV and *C. amaranticolor* defense system were studied using the qPCR technique. Different primer sets (Table 1) specific to pathogenesis-related protein-1 (PR-1), chalcone synthase (CHS), hydroxycinnamoyl-CoA quinate transferase (HQT), and TMV coat protein (CP) genes were used in this study. The housekeeping gene *β*-actin (Table 1) was used as a reference gene for the normalization of the transcript expression levels. The qPCR efficiency was determined for each gene and was between 93% and 100% for all genes. Each sample in all reactions was run in triplicate on a Rotor-Gene 6000 (QIAGEN, ABI System, Hilden, Germany) using the SYBR Green PCR Master Mix (Fermentas, Waltham, MA, USA) [58]. The single and discrete peak of the melting curve analysis at 55–95 °C confirmed the single amplified product for all genes. The amplification programs and the relative expression ratios were accurately quantified and calculated, as described previously [59,60]. Relative expression levels of more than 1 demonstrate an increase in accumulation (i.e., up-regulation), while values lower than 1 show a decrease in expression (i.e., down-regulation).

Table 1. Nucleotide sequences of the qRT-PCR primers used in this study.

Primer Name	Abbreviation	Direction	Sequence (5′–3′)	References
Pathogenesis-related protein-1	*PR-1*	Forward Reverse	CCAAGACTATCTTGCGGTTC GAACCTAAGCCACGATACCA	[61]
Chalcone synthase	*CHS*	Forward Reverse	CACCGTGGAGGAGTATCGTAAGGC TGATCAACACAGTTGGAAGGCG	[62]
Hydroxycinnamoyl Co A quinate hydroxycinnamoyl transferase	*HQT*	Forward Reverse	CCCAATGGCTGGAAGATTAGCTA CATGAATCACTTTCAGCCTCAACAA	[62]
Beta-actin	*β-actin*	Forward Reverse	ATGCCATTCTCCGTCTTGACTTG GAGTTGTATGTAGTCTCGTGGATT	[63]
Tobacco mosaic virus-coat protein	*TMV-CP*	Forward Reverse	ACGACTGCCGAAACGTTAGA CAAGTTGCAGGACCAGAGGT	[64]

2.7. Statistical Analysis

The relative expression levels of the antivirus activity data were analysed by one-way analysis of variance (ANOVA) using CoStat software. At the same time, significant differences were determined according to the least significant difference (LSD) $p \leq 0.05$ level of probability, and the standard deviation (SD) is shown as a column bar. Compared to the controls, relative expression levels higher than 1 demonstrated an increase in gene expression (i.e., up-regulation), while values lower than 1 showed a decrease in expression levels (i.e., down-regulation). Data of the antifungal property (i.e., the inhibition percentage of fungal linear growth) as affected by the tested concentrations (1%, 2%, and 3%) compared to the positive and negative controls were statistically analyzed using one-way ANOVA and processed with the Statistical Analysis Software (SAS) system [65]. The differences among the mean of the treatments were recorded using $LSD_{0.05}$.

3. Results

3.1. Polyphenolic Compounds in the Ethanol Extract

Table 2 shows the polyphenolic compounds found in the ethanolic WPE of *H. tuberculatum*. The main polyphenolic compounds were resveratrol (5178.58 mg/kg), kaempferol (1735.23 mg/kg), myricetin (561.18 mg/kg), rutin (487.04 mg/kg), quercetin (401.04 mg/kg), and rosmarinic acid (387.33 mg/kg).

Table 2. Polyphenolic compounds identified in the ethanol extract of the *Haplophyllum tuberculatum* whole plant by High Performance Liquid Chromatography (HPLC).

Compound	Amount (mg/kg)
Chlorogenic acid	ND
p-Coumaric acid	ND
Naringenin	ND
Pyrogallol	ND
Gallic acid	8.35
Ferulic acid	26.86
Catechin	27.43
Quinol	33.85
Syringic acid	35.91
Caffeic acid	39.63
Vanillic acid	45.14
Ellagic acid	45.39
Cinnamic acid	46.79
o-Coumaric acid	81.22
Catechol	120.66
Benzoic acid	199.51
p-Hydroxy benzoic acid	221.47

Table 2. *Cont.*

Compound	Amount (mg/kg)
Rosmarinic acid	387.33
Quercetin	401.04
Rutin	487.04
Myricetin	561.18
kaempferol	1735.23
Resveratrol	5178.58

ND, not detected.

3.2. Antifungal Property

Figure 1 shows the inoculated wood treated with the tested concentrations (1%, 2%, and 3%) prepared from the *H. tuberculatum* WPE with the two fungi *F. culmorum* and *R. solani*. It can be seen from the Petri dishes that with an increase in the extract concentration from 1% to 3%, fungal linear growth was suppressed. In addition, the positive control (25 µg of fluconazole) showed some inhibition in the growth of the tested fungi, while complete growth was recorded in the negative control (10% DMSO). The results of the inhibition percentage of fungal linear growth (IPFLG) are presented in Table 3. *H. tuberculatum* WPE (3%) followed by *H. tuberculatum* WPE (2%) showed the highest IPFLGs of 82.96% and 72.96%, respectively, against *F. culmorum* and were higher than 25 µg of fluconazole (53.70%). *H. tuberculatum* WPE at 3%, 2%, and 1% showed the highest IPFLGs against *R. solani* with values of 93.70%, 66.29%, and 49.62%, respectively, which were more elevated than the value from 25 µg of fluconazole (42.96%).

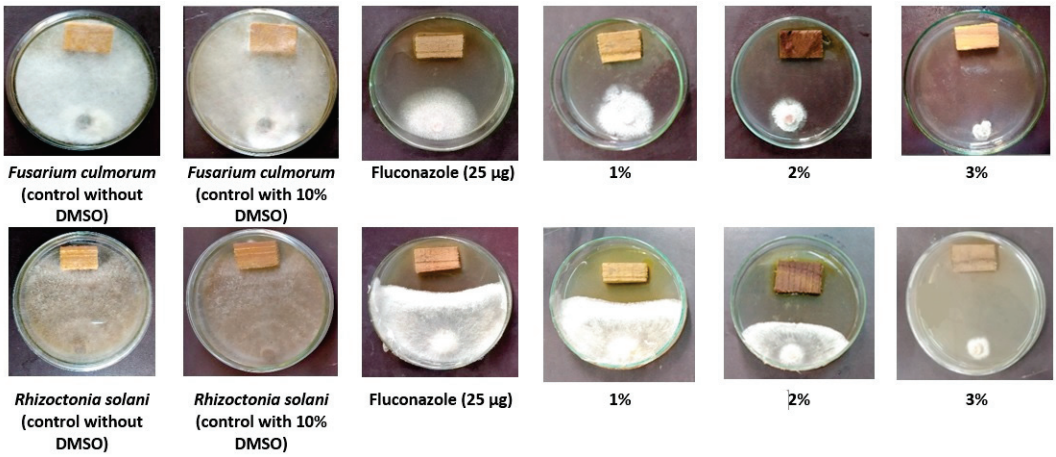

Figure 1. Visual observation of the antifungal property of *Haplophyllum tuberculatum* whole plant extract (WPE) against *Fusarium culmorum* and *Rhizoctonia solani*.

Table 3. Antifungal property of wood treated with *H. tuberculatum* WPE against the growth of *F. culmorum*, and *R. solani*.

Treatment	Inhibition Percentage of Fungal Linear Growth (%)	
	F. culmorum	*R. solani*
H. tuberculatum WPE (1%)	46.29d ± 0.37 *	49.62c ± 0.37
H. tuberculatum WPE (2%)	72.96b ± 0.37	66.29b ± 0.37
H. tuberculatum WPE (3%)	82.96a ± 0.37	93.70a ± 0.37
Fluconazole (25 µg)	53.70c ± 0.37	42.96d ± 0.37
Control (DMSO 10%)	0.00e	0.00e
LSD 0.05	1.04	1.04

* Values are reported as means ± standard error (SE). Means with the letter within the same column are not significantly difference according to LSD0.05. DMSO: Dimethyl sulfoxide.

3.3. Effect of H. tuberculatum WPE on Disease Severity and TMV Accumulation Levels

Under greenhouse conditions, the application of *H. tuberculatum* WPE (200 μg/mL) to *C. amaranticolor* plants significantly reduced the disease severity and decreased the TMV accumulation levels when compared to non-treated plants. The inhibitory effects of *H. tuberculatum* WPE were calculated by comparing the number of developed local lesions on the inoculated leaves at 5 dpi. In the protective assay, the calculated numbers of the local lesions on treated leaves (24 h before virus challenge) were significantly lower than that on non-treated leaves (Figure 2).

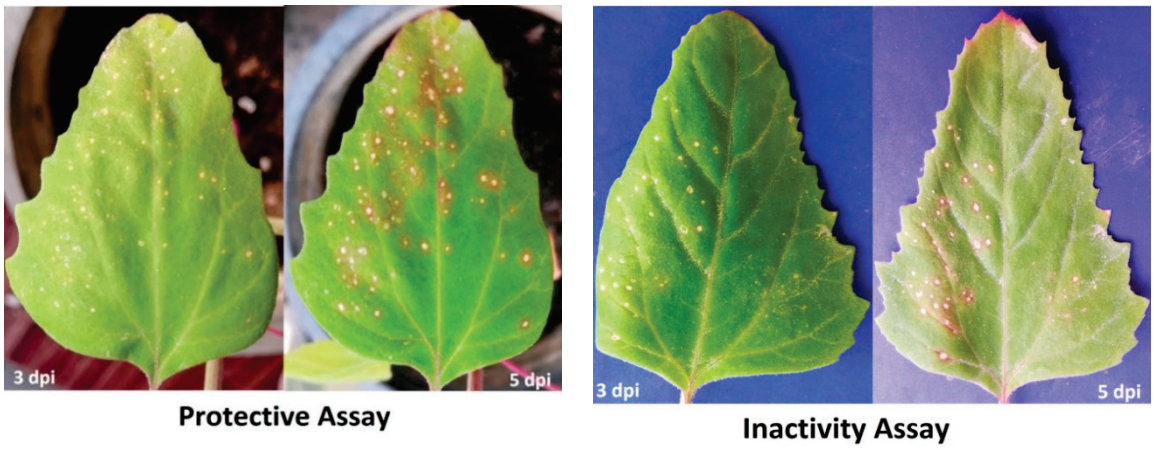

Figure 2. A photograph showing the disease symptoms on *Chenopodium amaranticolor* leaves infected with tobacco mosaic virus (TMV) at 3 and 5 days post-inoculation (dpi) of the protective activity and inactivity of *H. tuberculatum* whole plant extract (WPE) (200 μg/mL). The left-hand sides of the leaves were inoculated with TMV without any treatment, while the right-hand sides of the leaves were treated with WPE.

Moreover, the *H. tuberculatum* WPE showed an inhibitory effect of 65.38 ± 2.4%. On the other hand, the inactivity assay showed a higher inhibitory effect against TMV infection, with an inhibition rate of 95.73 ± 1.2% (Figure 3). No symptoms were observed on the mock-treated plants. Meanwhile, by using a specific primer of TMV-CP, the level of TMV-CP transcripts significantly decreased in *H. tuberculatum* WPE-treated plant tissues when compared to non-treated tissues. Compared to mock tissues at 5 dpi, the non-treated tissues showed higher accumulation levels of TMV with relative accumulation levels of 28.918- and 27.042-fold change for the protective activity and inactivity treatments, respectively. Notably, *H. tuberculatum* WPE-treated tissues exhibited a considerably decreased TMV concentration level. Compared to the controls, the inactivity and protective activity treatments showed TMV accumulation levels of 2.470- and 3.499-fold change, respectively (Figure 3).

3.4. Protective Assay: Changes in the Transcriptional Levels of PR-1, CHS, and HQT

Figure 4 shows significant increases in the relative expression levels of PR-1, CHS, and HQT in plants treated with *H. tuberculatum* WPE when compared to that in non-treated plants ($p \leq 0.05$) at 3 and 5 dpi. Compared to the controls, a significant up-regulation of PR-1 with relative expressions of 1.926- and 7.467-fold change were observed in non-treated tissues at 3 and 5 dpi, respectively. However, *H. tuberculatum* WPE-treated tissues exhibited overexpression of PR-1 with relative expression levels of 12.436- and 14.750-fold change at 3 and 5 dpi, respectively, compared to the controls. For the CHS transcripts, at 3 dpi, up-regulation with a significant relative expression level of 1.778-fold change was observed in *H. tuberculatum* WPE-treated tissues. In contrast, down-regulation with a relative expression level of 0.359-fold change was observed in non-treated tissues when compared to the control tissues. On the other hand, up-regulation with relative expression levels of 1.880- and 2.512-fold change were showed in non-treated and *H. tuberculatum* WPE-treated tissues, respectively, at 5 dpi compared to the controls. Concerning the HQT gene, significant up-regulation with relative expression levels of

1.340- and 1.573-fold change were shown only in *H. tuberculatum* WPE-treated tissues at 3 and 5 dpi, respectively, when compared to the controls. The down-regulation of HQT with relative transcriptional levels of 0.502- and 0.913-fold change lower than the controls was observed in non-treated tissues at 3 and 5 dpi, respectively. Consequently, treatment of *C. amaranticolor* tissues with *H. tuberculatum* WPE 24 h before TMV challenge induced the expression of HQT. However, TMV induced the expression of PR-1 and CHS, while the *H. tuberculatum* WPE applications triggered the expression of both genes at 3 and 5 dpi.

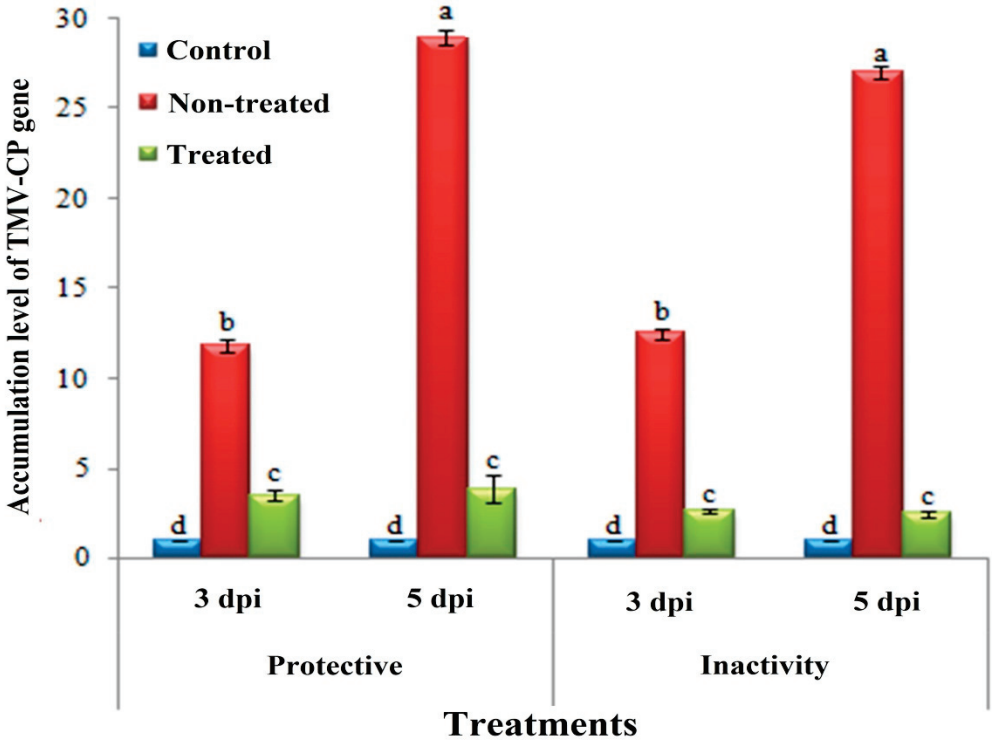

Figure 3. A histogram showing the accumulation levels of the TMV-CP gene at 3 and 5 days dpi with the protective activity and inactivity of *H. tuberculatum* WPE treatments (200 µg/mL). Control = mock-treated plants; non-treated = plants inoculated with TMV only without any treatment; treated = plants treated with WPE, 24 h before inoculation of TMV for the protective assay and 24 h after inoculation of TMV for the inactivity assay. Columns represent a mean value from three biological replicates and the bars indicate the standard deviation (SD). Significant differences between samples were determined by one-way analysis of variance (ANOVA) using CoStat software. Means were separated by the least significant difference (LSD) test at $p \leq 0.05$ and indicated by lowercase letters. Columns with the same letter do not differ significantly.

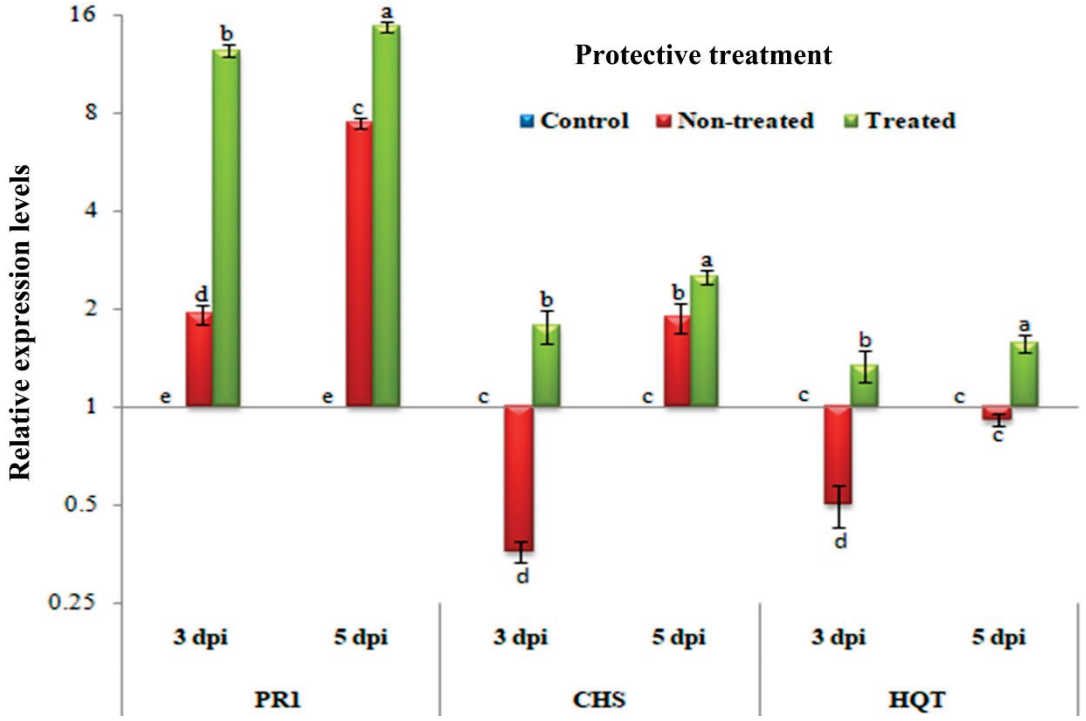

Figure 4. A histogram showing the relative expression levels of the PR-1, CHS, and HQT genes at 3 and 5 dpi of *H. tuberculatum* WPE treatments (200 µg/mL) in the protective activity assay. Control = mock-treated plants; non-treated = plants inoculated with TMV only without any treatment; treated = plants treated with *H. tuberculatum* WPE, 24 h before inoculation of TMV for the protective assay and 24 h after inoculation of TMV for the inactivity assay. Columns represent the mean value from three biological replicates and the bars indicate SD. Significant differences between samples were determined by one-way ANOVA using CoStat software. Means were separated by the LSD test at $p \leq 0.05$ and indicated by lowercase letters. Columns with the same letter do not differ significantly.

3.5. Inactivity Assay: Changes in Transcriptional Levels of PR-1, CHS, and HQT

In Figure 5, similarly to the protective treatment, significant increases in the relative expression levels of PR-1, CHS, and HQT were observed in plant tissues treated with *H. tuberculatum* WPE when compared to the control and non-treated plants ($p \leq 0.05$) at 3 and 5 dpi. Compared to the mock tissues, a significant up-regulation of PR-1 with relative expression levels of 2.162- and 3.342-fold change was observed in non-treated tissues at 3 and 5 dpi, respectively. However, *H. tuberculatum* WPE-treated tissues showed an increase in the transcription of PR-1 with relative expression levels of 12.036- and 15.763-fold change at 3 and 5 dpi, respectively, compared to the controls. Regarding the CHS gene, down-regulation with a relative expression level of 0.815-fold change was observed in non-treated tissues at 3 dpi. In contrast, *H. tuberculatum* WPE-treated tissues showed up-regulation with a significant relative expression level of 1.848-fold change at the same time when compared to the control tissues. Subsequently, at 5 dpi, an increase in the expression with relative expression levels of 3.215- and 3.172-fold change was observed in non-treated and *H. tuberculatum* WPE-treated tissues, respectively. For the HQT transcripts, significant up-regulation with relative transcriptional levels of 1.470- and 1.401-fold change were found only in *H. tuberculatum* WPE-treated tissues at 3 and 5 dpi, respectively, when compared to the controls. Conversely, the non-treated tissues exhibited down-regulation of HQT with relative transcriptional levels of 0.603- and 0.795-fold change lower than the controls at 3 and 5 dpi, respectively.

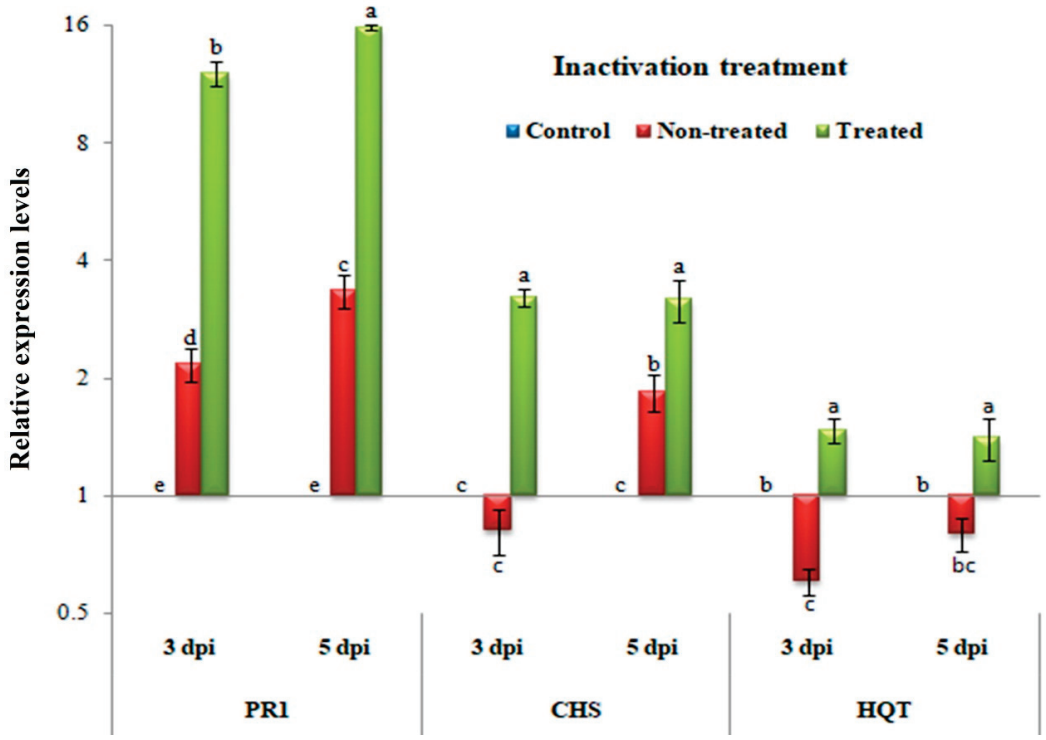

Figure 5. A histogram showing the relative expression levels of the PR-1, CHS, and HQT genes at 3 and 5 dpi of *H. tuberculatum* WPE treatments (200 µg/mL) in the inactivity assay. Control = mock-treated plants; non-treated = plants inoculated with TMV only without any treatment; treated = plants treated with *H. tuberculatum* WPE, 24 h before inoculation of TMV for the protective assay and 24 h after inoculation of TMV for the inactivity assay. Columns represent a mean value from three biological replicates and bars indicate SD. Significant differences between samples were determined by one-way ANOVA using CoStat software. Means were separated by the LSD test at $p \leq 0.05$ and indicated by lowercase letters. Columns with the same letter do not differ significantly.

4. Discussion

Several polyphenolic compounds from the ethanolic WPE of *H. tuberculatum* were identified by HPLC, such as resveratrol, kaempferol, myricetin, rutin, quercetin, rosmarinic acid, catechol, *p*-hydroxybenzoic acid, and benzoic acid. The *H. tuberculatum* WPE showed the presence of total phenol content (TPC) ranging between 0.27 and 11.97 mg gallic acid equivalent (GAE)/g dry matter and a whole flavonoid content from 0.05 to 1.50 mg equivalent of rutin/g of dry matter [66]. The TPC was 46.2 mg GA/g sample, and the main chemical constituents of quercetin derivatives, cinnamic acid, ferulic acid, vanillic acid, and benzoic acid were found in the ethanol extract of the aerial parts of *H. tuberculatum* [67]. The TPC was observed to be 561.22 mg/g of GAE and the flavonoids 165.54 mg/g of quercetin equivalent [68]. The ethyl acetate extract of *H. tuberculatum* leaves was the most abundant extract in phenolics and flavonoids, with 262 mg GAE/g and 99.1 mg quercetin equivalent/g of dry weight, respectively [42].

In the present study, all of the examined *H. tuberculatum* WPE concentrations exhibited antifungal properties against the linear growth of two fungal isolates compared with the positive control (fluconazole), which commercially used in a rapid susceptibility testing useful method to determine the optimal treatment for infection with resistant isolates [69].

Many strategies have been used to reduce agricultural losses caused by fungal diseases including spraying of chemicals, biological control [70], and azoles fungicide [71]. The azoles group gave high minimal inhibitory concentrations (MICs) against the most *Fusarium* species [72]. *Candida albicans* is usually acutely susceptible to fluconazole; fluconazole MICs for approximately 90% of *C. albicans*

isolates are ≤1 μg/mL [73]. Some non-*C. albicans* yeasts have been noted to have decreased susceptibility or resistance to fluconazole [73].

Our recent research similarly showed the highest inhibition of *R. solani*, *B. cinerea*, and *F. culmorum* growth by 64.4%, 100%, and 38.5%, respectively, with the ethanol extract of *Coccoloba uvifera* L. at 3% [48]. The *Eucalyptus camaldulensis* L. aerial parts n-hexane extract showed the same strong fungicidal property against the two fungal isolates, *F. culmorum* and *R. solani* especially at the concentration of 3% [50]. In the same way, wood samples treated with *Acacia saligna* water extract showed inhibition of fungal mycelial growth of *F. culmorum* and *R. solani* [15]. In a study performed by Sabry et al. [74] the ethanolic extract of the aerial parts of *H. tuberculatum* demonstrated an efficient antifungal property against *Aspergillus fumigates*, *Geotricum candidum* and *Syncephalastrum racemosum* with (MIC 0.49, 0.12, and 1.95 μg/mL). While the antimicrobial tests of *H. tuberculatum* extracts were more effective against Gram-negative bacteria than Gram positive ones. The best antibacterial activity was exhibited by methanolic extract, which was also active against *C. albicans* [75].

Other works reported the cytotoxicity of the extracted parts of *H. tuberculatum* on other pests, and the hexane, chloroform, ethyl acetate, butanol, methanol, and water extracts of the leaves of *H. tuberculatum* displayed significant cytotoxic activity against brine shrimp larvae. At the same time, the ethanol extract of the aerial parts of *H. tuberculatum* has shown good insecticidal activity against *Culex quinquefasciatus* [38]. In comparison, the oil of *H. tuberculatum* has been observed to have a slightly antimicrobial effect on the growth of *Escherichia coli*, *Salmonella choleraesuis*, and *Bacillus subtilis*, as well as antifungal activity against *C. lunata* and *F. oxysporum* growth. Still, it does not affect the germination of their spores [40]. In a different way the fungicidal property of the *H. tuberculatum* might be went to its composition of flavonoids, tannins, phenolic acids, especially resveratrol, which displays better antifungal than antibacterial activity, as demonstrated by the minimum inhibitory concentrations (MICs). For the fungal species *C. albicans*, *Saccharomyces cerevisiae* and *Trichosporon beigelii*, the inhibitory activity is 10–20 μg/mL [76]. Resveratrol displays inhibitory activity against the plant pathogen *B. cinerea*, the causal agent of grey mold, where reduced germination of *B. cinerea* conidia and mycelial growth is observed at concentrations of 60–140 μg/mL [77]. While for the antibacterial activity the resveratrol exhibited MIC > 400 against the Gram negative bacteria, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas aeruginosa* [78]. In our study, anti-TMV, protective, and inactivating, the activity of *H. tuberculatum* WPE on *C. amaranticolor* tissues using the half-leaf method [53] was investigated for the first time. Mainly, the inhibitory effects, accumulation levels of TMV CP, and relative expression levels of three defense-related genes (i.e., PR-1, CHS, and HQT) at 3 and 5 dpi were evaluated. Overall, our results indicated that *H. tuberculatum* WPE had an inhibitory effect against TMV infection. In the current study, the application of *H. tuberculatum* WPE (200 μg/mL) showed a significant reduction in local lesion symptoms when *C. amaranticolor* tissues were treated either 24 h before or with to viral challenge. The inactivity of *H. tuberculatum* WPE exhibited a strong inhibitory effect (approximately 96%), while the protective activity showed an inhibitory effect of 65%. The treatment of TMV with the aqueous extract of *Bryophyllum daigremontianum* (200 mg/mL) before mechanical inoculation significantly reduced the number of local lesions in *N. tabacum* var. *Xanthi*, *N. glutinosa*, and *V. faba* plants and showed inhibitory effects ranging from 51.45% to 86.08% [79]. The qPCR results confirmed the antiviral activity of *H. tuberculatum* WPE against TMV infection, which resulted in a considerable decrease in the viral accumulation level inside the treated tissues. The relative accumulation levels of TMV CP in *C. amaranticolor* tissues were 3.866- and 2.470-fold change in the protective activity and inactivity treatments, respectively, while non-treated tissues exhibited 28.918- and 27.042-fold change, respectively, at 5 dpi. These results suggest that *H. tuberculatum* WPE can directly inactivate TMV and may interfere with coat proteins or may inhibit viral replication inside plant cells. Jing et al. [31] reported that several plant extracts inhibited TMV infection through preventing the infection or spread of TMV, as well as the inhibition of viral replication.

In general, the direct and indirect inhibition of viral replication, through simultaneous activation of the host's innate immune system and by inducing SAR against viral infection, are two mechanisms

of antiviral agents [31,37]. Regarding the stimulating effect on *C. amaranticolor* tissues, *H. tuberculatum* WPE induced and activated the expression of three defense-related genes (i.e., PR-1, CHS, and HQT).

PR-1 is considered a principal regulator of SAR and could be a marker of plant early defense responses [80]. Moreover, salicylic acid (SA) is a vital signal phytohormone molecule of SAR in plants [81], and its role in plant immunity has been known for over two decades. The activation of SA in response to pathogens is associated with the accumulation and expression of PR-1 as a SA marker gene [59]. In the present study, the non-treated *C. amaranticolor* tissues challenged with TMV showed induction of PR-1 with relative expression levels of 1.926- and 7.467-fold change and 2.162- and 3.342-fold change in protective activity and inactivity treatments at 3 and 5 dpi, respectively. However, the *H. tuberculatum* WPE-treated tissues exhibited overexpression of PR-1 with transcriptional levels of 12.436- and 12.036-fold change in protective activity and inactivity treatments, respectively, at 3 dpi. At 5 dpi, PR-1 continued to accumulate, reaching maximum levels of 14.750- and 15.763-fold change in protective activity and inactivity treatments, respectively, when compared to the controls. Consequently, we suggest that *H. tuberculatum* WPE may contain elicitor molecules that activate the immune defense system besides the inhibition of TMV replication. In this context, tobacco plants treated with *Sophora flavescens*, *Forsythia suspense*, and *Lonicera japonic* extracts exhibiting the induction and up-regulation of PR-1 resulted in the development of SAR against TMV [82].

Besides, as the first enzyme in the flavonoid pathway that catalyzes the synthesis of naringenin chalcones, CHS is strictly required in various plant tissues for flavonoid production [59,83]. Compared to mock tissues at 3 dpi, the CHS transcripts were induced only in *H. tuberculatum* WPE-treated tissues with relative expression levels of 1.778- and 3.215-fold change for protective activity and inactivity treatments, respectively. At 5 dpi, up-regulation of CHS in non-treated tissues was observed, while *H. tuberculatum* WPE-treated tissues exhibited an increase in the transcriptional levels of CHS. The down-regulation of CHS at 3 dpi of non-treated tissues suggests that TMV infection suppresses naringenin chalcones biosynthesis in early infection. Interestingly, the application of *H. tuberculatum* WPE in the protective activity and inactivity assays showed the highest induction of CHS that is strictly required for flavonoid production naringenin chalcones, which are considered the primary precursors and constitute the main intermediates for the synthesis of many flavonoids by the action other enzyme sets [8,84].

Chlorogenic acid (CGA), one of the most polyphenolic compounds, plays important roles in increasing plant resistance and in inhibiting pathogens, including viruses [85–87]. HQT is the key enzyme in the biosynthesis of CGA, while it catalyzes caffeoyl-CoA and quinic acid to form CGA [88]. In the present study, the transcription of CHS was wholly suppressed and down-regulated in non-treated tissues at 3 and 5 dpi of the protective activity and inactivity treatments when compared to the controls. The overexpression of HQT was associated with increases in chlorogenic acid content and versa [88]. Consequently, TMV was able to suppress chlorogenic acid biosynthesis inside infected tissues.

On the other hand, the application of *H. tuberculatum* WPE induced HQT transcripts in both treatments, i.e., protective activity and inactivity, at 3 and 5 dpi. A higher expression level of HQT (1.573-fold change) was shown in *H. tuberculatum* WPE-treated tissues of the protective activity assay at 5 dpi. In comparison, a high expression level of HQT in the inactivity assay (1.470-fold change) was observed at 3 dpi. Based on the current results, *H. tuberculatum* WPE induced and activated HQT transcripts that correlated with increasing CGA accumulation inside treated tissues.

5. Conclusions

We firstly examined *H. tuberculatum* WPE as a novel antiviral agent against plant viruses, and our results suggest that it contains compounds that penetrate plant cells, play significant roles in SAR, inhibit infection, and directly inactivate TMV. Consequently, *H. tuberculatum* WPE may be considered as a promising source of both antifungal and antiviral substances for practical use and for developing plant-derived compounds for the effective management of plant diseases.

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Polyphenol Profile and Biological Activity Comparisons of Different Parts of *Astragalus macrocephalus* subsp. *finitimus* from Turkey

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Abstract: The members of the genus *Astragalus* have great interest as traditional drugs in several folk systems including Turkey. In this sense, the present paper was aimed to explore the biological properties and chemical profiles of different parts (aerial parts, leaves, flowers, stems, and roots) of *A. macrocephalus* subsp. *finitimus*. Antioxidant (radical quenching, reducing power, and metal chelating) and enzyme inhibitory (α-amylase and tyrosinase) effects were investigated for biological properties. Regarding chemical profiles, individual phenolic compounds were detected by LC-MS, as well as total amounts. The leaves extract exhibited the strongest antioxidant abilities when compared with other parts. However, flowers extract had the best metal chelating ability. Hyperoside, apigenin, p-coumaric, and ferulic acids were identified as main compounds in the tested parts. Regarding enzyme inhibitory properties, tyrosinase inhibitory effects varied from IC_{50}: 1.02 to 1.41 mg/mL. In addition, the best amylase inhibition effect was observed by leaves (3.36 mg/mL), followed by aerial parts, roots, stems, and flowers. As a result, from multivariate analysis, the tested parts were classified in three cluster. Summing up the results, it can be concluded that *A. macrocephalus* subsp. *finitimus* could be a precious source of natural bioactive agents in pharmaceutical, nutraceutical, and cosmeceutical applications.

Keywords: *astragalus*; antioxidant; α-amylase; hyperoside; bioactive compounds

1. Introduction

Since the beginning of the last century, scientists have been focused on the biological and chemical properties of plants with ethnobotanical evidence [1–3]. From their studies, several important compounds have been introduced. As a springboard, the ethnobotanical records in ancient times indicated that *Artemisia annua* had great potential against malaria. In the light of this information, Japanese and Chinese scientist have isolated one sesquiterpene (artemisinin) from this plant to combat malaria, which they won the Nobel Prize for in 2015 [4,5]. In this sense, traditional and scientific data have to combine for further applications. Turkey has significant ethnobotanical data, with remarkable floristic features (about 12,000 plants) [6]. However, most of them have been scarcely investigated. Thus, the uninvestigated plants could be considered a treasure for pharmaceutical and medicinal applications.

In the last decade, plant secondary metabolites, especially phenolic compounds, have been gaining interest in the scientific platform. These compounds contain one or more hydroxyl groups and they have good hydrogen/electron donating abilities. Thus, these compounds are considered as main contributors to antioxidant properties. Additionally, these compounds have a broad spectrum of biological activities such as antimicrobial, anti-inflammatory, and anti-cancer [7,8].

The genus *Astragalus* is one of the biggest genera in the family Fabaceae and is represented by more than 2500 species [9]. The genus also contains 478 taxa in Turkey and it has many endemic species (202,

endemism ratio: 42%) to Turkey [10]. Regarding folk medicinal uses, the genus is traditionally used for several purposes. For example, *A. gumnifer* and *A. longifolius* roots are used to treat diabetes mellitus [11]. Additionally, *A. aureus* and *A. brachylcalyx* are used against stomachache and sore throat [12]. In addition, *A. lamarckii* for ulcer [13]; *A. cephalotes* var. *brevicalyx* for wound healing [14] and *A. tmoleus* for abdominal pain and toothache [15]. From the light of these ethnobotanical records, several biological and chemical studies were performed on the members of the genus [16–22]. In the chemical studies, some biologically-active compounds, including hyperoside, apigenin, kaempferol, and naringenin, were detected [9]. However, to the authors best knowledge, very few publications can be found biological properties of *Astragalus microcephalus* [23–25]. *A. microcephalus* is a stout and erect perennial plant (50–100 cm). Leaves are lanceolate and narrowly elliptic. Inflorescence is 3.5–5 mm diameter and contains 30–50 sessile flowers. Calyx is 15–18 mm and tubular-campanulate. Corolla is 18–35 mm and deep yellow [26]. In the current work, we aimed to examine biological properties (antioxidant and enzyme inhibitory effect) and chemical composition (total and individual phenolic compounds) of different parts (aerial parts, leaves, flowers, stems and roots) of *A. macrocephalus* subsp. *finitimus*.

2. Materials and Methods

2.1. Plant Material and Solvent Extraction

Astragalus macrocephalus Willd. subsp. *finitimus* (Bunge) Chamberlein (Fabaceae) were collected from Sucati village, Gurun, Sivas-Turkey on 23 June, 2019 (1351 m, 38°43'15.06" N 37°21'43.22" E), authenticated by Olcay Ceylan, and deposited (AD-1518) at the Department of Biology, Mugla Sıtkı Koçman University (Mugla, Aegean, Turkey). The plant was collected in the flowering season and the aerial parts do not contain fruit and seeds. The plant was firstly divided into different parts (aerial parts (as mix leaves, flowers, and stems) roots, leaves, flowers, and stems). The plant materials were dried in a shaded and well-ventilated environment (about 10 days) and were powdered in a laboratory mill. After powdering process, the plant materials were used to obtain extracts in the same week.

The methanol extracts from different parts of *A. macrocephalus* subsp. *finitimus* were prepared by maceration for 24 h. Five grams of different parts (aerial parts, roots, leaves, flowers, and stems) were mixed with 100 mL of solvent (the ratio of solid/solvent: 1:20) and agitation was set to 150 rpm in dark environment at room temperature. All of the extracts were stored at +4 °C until analyzed after concentrating the methanol extracts under reduced pressure. Extraction yields were given in Table 1.

Table 1. Extraction yield, total phenolic and flavonoid contents of the methanol extracts from different parts of *A. macrocephalus* subsp. *finitimus* [x].

Samples	Yield (%)	Total Flavonoids (Mg QE/g Extract)	Total Phenolics (Mg GAE/g Extract)
Aerial parts	10.56	21.06 ± 0.11 ^{c}	10.01 ± 0.17 ^{b}
Flowers	6.95	29.90 ± 0.95 ^{b}	6.96 ± 1.08 ^{bc}
Leaves	3.46	39.23 ± 1.64 ^{a}	37.68 ± 0.74 ^{a}
Roots	17.83	6.03 ± 0.05 ^{d}	5.60 ± 0.06 ^{c}
Stems	11.78	7.91 ± 0.37 ^{d}	8.29 ± 1.13 ^{bc}

[x] Within each column, means sharing the different superscripts (a–d) show comparison between the extracts using Tukey's test at $p < 0.05$, GAEs and QEs, gallic acid and quercetin equivalents, respectively.

2.2. Total Flavonoid and Phenolic Contents

To obtain total level of phenolic (TPC) and flavonoid content (TFC) in the extracts, colorimetric assays were used as described in our previous paper [27]. Gallic acid (GAE) and quercetin (QE) were used as standards, respectively. Please see the Supplementary Materials for the details.

2.3. Liquid Chromatography–Electrospray Tandem Mass Spectrometry (LC–ESI–MS/MS) Analysis

To determine chemical compositions in the extracts, we used an Agilent Technologies 1260 Infinity liquid chromatography system (Santa Clara, CA, USA) hyphenated to a 6420 Triple Quad mass spectrometer on which a chromatographic separation on a Poroshell 120 EC-C18 (100 mm × 4.6 mm I.D., 2.7 μm) column [28]. All analytical and chromatographic details are given in the Supplementary Materials. The different analytes were identified by means of their retention times, mass spectra, and tandem mass spectra. Specifically, quantitative analyses were performed using a specific MRM transition for each analyte. Analytical parameters and chromatograms are given in supplemental materials (Table S1 and Figure S1).

2.4. Biological Activity

Antioxidant properties of these extracts were detected by several assays including DPPH radical [29] $ABTS^+$ free radical scavenging [30], cupric ion (CUPRAC) and ferric ion (FRAP) reducing power [31,32], phosphomolybdenum method [33] and ferrous ion chelating [34]. The antioxidant properties were evaluated by IC_{50} values (the half inhibitory concentration). The IC_{50} values were calculated from the graph of percentage ($ABTS^+$, DPPH and metal chelating) against the concentration of the extracts. IC_{50} values for other assays (reducing power and phosphomolybdenum) reflect that the concentration at which absorbance is 0.5. For this purpose, we used the graph of absorbance against the concentration of the extracts. Trolox (TE) and Ethylenediaminetetraacetic acid (disodium salt) (EDTA)) were used as positive controls. In addition, the results were expressed as equivalents of these standards.

The key enzymes inhibition activity of the extracts against tyrosinase, and α-amylase were measured using the protocols as published by [35]. The enzyme inhibition abilities were evaluated by IC_{50} values. IC_{50} values calculated as antioxidant assays and we used a graph between concentration and percentage of enzyme inhibition. Standard enzyme inhibitors (Kojic acid (KAE) for tyrosinase and acarbose (ACE) for α-amylase) were used as positive control and also, the results were expressed as equivalents of these standards. The details for experimental methods are given in the Supplementary Materials.

2.5. Statistical Analysis

Obtained results were given as mean ± standard deviation (SD) and the results were evaluated by ANOVA assay (with Tukey's test, significant value: $p < 0.05$). Principal component analysis (PCA) and hierarchical clustered analysis (HCA) were applied to the experimental data under FactoMineR (Factor Analysis and Data Mining with R) package (R Core Team, Vienna, Austria). The antioxidant activities of the extracts were analyzed using various methods. As is well known, each of the antioxidant activity methods has a different mechanism of action on the extracts. Therefore, it is not possible to directly compare the results obtained with each other. Relative antioxidant capacity (RACI) index values were calculated to make the results comparable, and the correlation between the results obtained from each test and RACI values were presented separately [36]. The RACI values of the samples were determined for each test by dividing into standard deviation after subtracting these mean values from the raw data. Total RACI values were calculated by averaging the RACI values obtained from all antioxidant tests of the relevant sample (including phenolic and flavonoid).

3. Results and Discussion

3.1. Phytochemical Composition

The amounts of total phenolics and flavonoids in the tested extracts were affected by plant parts used. As shown in Table 1, the highest levels of phenolics and flavonoids were determined in the leaves extract (37.68 mg GAE/g and 39.23 mg QE/g). Flowers (6.96 mg GAE/g) and roots (6.03 mg QE/g) had the lowest level of total phenolics and flavonoids, respectively. Several studies reported different levels of these compounds in the members of the genus *Astragalus* [16,37–39]. Observed differences may be linked with geographical, environmental, and climatic conditions as well as plant parts [37,40–42]. In addition,

recent studies indicated that the colorimetric methods had several drawbacks and these methods could not reflect accurate levels of these compounds in plant extracts [43,44]. Hence, chromatographic methods such as HPLC or LC-MS are required to provide certain data. In this context, the extracts were analyzed by LC-MS and the results are given in Table 2. Hyperoside, p-coumaric and ferulic acids and apigenin were identified as main compounds in the tested extracts. The level of hyperoside varied from 2.90 (in roots) to 1828.94 (in leaves) μg/g extract. The highest level of p-coumaric acid was detected in flowers extract with 146.78 μg/g extract. The main compounds in the extracts exhibited significant biological activities in earlier studies. For example, hyperoside is a main compound in the genus *Hypericum* and this compound exhibits promising biological abilities [45–47]. Additionally, similar properties were also reported for p-coumaric acid [48], apigenin [49] and ferulic acid [50,51]. From this point, observed biological activities of *A. macrocephalus* subsp. *finitimus* extracts might be linked to the presence of these compounds.

Table 2. Concentration (μg/g extract) of selected phytochemicals in the methanol extracts from different parts of *A. macrocephalus* subsp. *finitimus* [x].

Compounds	Aerial Parts	Flowers	Leaves	Roots	Stems
Gallic acid	2.28 ± 0.03 ^{d}	8.76 ± 0.18 ^{a}	2.24 ± 0.01 ^{d}	4.93 ± 0.12 ^{b}	3.03 ± 0.04 ^{c}
Protocatechuic acid	14.37 ± 0.08 ^{c}	16.80 ± 0.47 ^{b}	18.90 ± 0.16 ^{a}	13.51 ± 0.64 ^{c}	8.06 ± 0.05 ^{d}
Chlorogenic acid	8.99 ± 0.24 ^{a}	10.29 ± 4.02 ^{a}	0.75 ± 0.26 ^{b}	3.06 ± 0.69 ^{ab}	1.59 ± 0.19 ^{b}
2,5-Dihydroxybenzoic acid	9.96 ± 1.74 ^{c}	nd	23.14 ± 0.06 ^{a}	nd	16.86 ± 1.09 ^{b}
4-Hydroxybenzoic acid	12.78 ± 0.16 ^{c}	28.83 ± 0.62 ^{b}	2.72 ± 0.07 ^{e}	31.86 ± 0.76 ^{a}	8.13 ± 0.28 ^{d}
(−)-Epicatechin	nd	nd	5.60 ± 0.11	nd	nd
Caffeic acid	2.77 ± 0.11 ^{b}	nd	3.94 ± 0.03 ^{a}	nd	nd
Vanillic acid	23.30 ± 0.23 ^{c}	100.53 ± 8.83 ^{a}	4.36 ± 0.26 ^{d}	57.78 ± 4.27 ^{b}	51.53 ± 3.73 ^{b}
Syringic acid	6.71 ± 0.03 ^{d}	20.89 ± 0.71 ^{b}	2.08 ± 0.06 ^{e}	33.47 ± 1 ^{a}	17.27 ± 1.52 ^{c}
Vanillin	3.42 ± 0.11 ^{c}	nd	nd	55.68 ± 0.68 ^{a}	23.62 ± 0.13 ^{b}
Verbascoside	43.48 ± 0.25 ^{a}	8.68 ± 0.30 ^{b}	1.13 ± 0.08 ^{e}	7.29 ± 0.14 ^{c}	2.64 ± 0.11 ^{d}
Sinapic acid	7.41 ± 0.01 ^{b}	52.66 ± 1.36 ^{a}	3.41 ± 0.52 ^{c}	nd	nd
p-Coumaric acid	76.85 ± 0.47 ^{b}	146.78 ± 1.89 ^{a}	33.12 ± 0.35 ^{c}	6.81 ± 0.60 ^{e}	22.12 ± 0.51 ^{d}
Ferulic acid	52.79 ± 2.88 ^{b}	64.20 ± 2.45 ^{a}	37.61 ± 0.30 ^{c}	10.53 ± 0.48 ^{d}	17.40 ± 0.96 ^{d}
Luteolin 7-glucoside	17.63 ± 0.89 ^{ab}	28.59 ± 9.16 ^{a}	13.45 ± 0.17 ^{ab}	6.20 ± 0.75 ^{b}	11.35 ± 0.16 ^{b}
Hesperidin	7.69 ± 0.04 ^{ab}	8.44 ± 1.45 ^{a}	7.05 ± 0.03 ^{ab}	4.99 ± 0.06 ^{b}	9.14 ± 0.75 ^{a}
Hyperoside	401.68 ± 6.72 ^{b}	90.45 ± 1.39 ^{d}	1828.94 ± 21 ^{a}	2.90 ± 0.46 ^{e}	321.43 ± 7.64 ^{c}
Rosmarinic acid	2.10 ± 0.11 ^{c}	26.95 ± 3.30 ^{a}	1.25 ± 0.02 ^{c}	10.46 ± 0.65 ^{b}	5.43 ± 0.27 ^{bc}
Apigenin 7-glucoside	23.68 ± 0.08 ^{c}	63.56 ± 1.94 ^{a}	16.85 ± 0.19 ^{d}	44.54 ± 0.35 ^{b}	20.24 ± 0.04 ^{cd}
Pinoresinol	nd	nd	nd	6.40 ± 0.37 ^{a}	6.80 ± 0.23 ^{a}
Eriodictyol	0.44 ± 0.05 ^{c}	2.58 ± 0.36 ^{b}	0.30 ± 0.01 ^{c}	6.97 ± 0.12 ^{a}	0.73 ± 0.01 ^{c}
Quercetin	6.47 ± 0.02 ^{b}	11.11 ± 0.04 ^{a}	3.27 ± 0.06 ^{d}	4.58 ± 0.13 ^{c}	4.74 ± 0.12 ^{c}
Luteolin	32.06 ± 0.45 ^{cd}	81.94 ± 0.28 ^{b}	32.58 ± 1.07 ^{c}	108.11 ± 1.20 ^{a}	28.77 ± 0.73 ^{d}
Kaempferol	1.58 ± 0.25	nd	nd	nd	nd
Apigenin	29.07 ± 0.50 ^{c}	52.33 ± 1.34 ^{b}	23 ±0.12 ^{c}	181.90 ± 6.23 ^{a}	42.81 ± 0.52 ^{b}

[x] Within each row, means sharing the different superscripts (a–d) show comparison between the samples using Tukey's test at $p < 0.05$. nd, not detected.

3.2. Antioxidant Properties

Oxidative stress is the main etiological factor for the progression of several chronic and degenerative diseases such as Alzheimer's disease, cancer, and cardiovascular diseases. Thus, the balance between the production of free radicals and the endogenous antioxidant defense system plays a pivotal role in healthy physiological function [52]. At this point, we need to support the defense system with dietary antioxidants. Plants are the main sources of the dietary antioxidants and several studies have reported a negative association between the consumption of plants and the frequency of these diseases [53–55]. In the present study, to evaluate the antioxidant effects of *A. macrocephalus* subsp. *finitumus* extracts, several chemical methods were performed, and their results are shown in Table 3. We used IC_{50} values and standard equivalents (trolox (TE) and EDTA (EDTAE)) to express antioxidant abilities. Based on Table 3, the strongest antioxidant abilities were detected in leaves extracts. For example, the lowest IC50 values were detected in the leaves extract for radical scavenging (ABTS and DPPH) and to reduce power

(FRAP, CUPRAC and phosphomolybdenum). Observed antioxidant effects for leaves extract could be explained with the high level of phenolics and we obtained a good correlation between these parameters Table 4. In accordance with our findings, several researchers reported a positive correlation between total phenolic content and antioxidant properties. Interestingly, the metal chelating abilities of the tested extracts can be ranked as flowers>stems>roots>aerial parts>leaves. In addition, a negative relationship was observed between total bioactive compounds (phenolics and flavonoids) and metal chelating ability. Taken together, we could imply that observed findings could be linked with the presence of non-phenolic chelators such as peptides, polysaccharides, and ascorbic acid. In earlier studies, several authors reported antioxidant properties of some *Astagalus* species such as *A. ponticus* [16], *A. lagurus* [56], *A. spruneri* [57], *A. membranaceus* [58,59]. With this in mind, the members of the genus *Astragalus* could be considered as valuable sources of natural antioxidants.

Several researchers suggested that only one method is not enough to evaluate antioxidant abilities of plant extracts and thus, multiple methods including different mechanisms are required to obtain a full antioxidant picture. [52,60]. However, different expression methods have been observed in these different methods. With this fact, any comparison between results might be unreasonable and sometimes impossible. Thus, relative antioxidant capacity index (RACI) has been developed by some researchers to obtain an accurate comparison between studies [36,61]. In the present study, we calculated the relative antioxidant capacity index for each part in Figure 1 and each method in Figure 2. Clearly, among the tested plant parts, the leaves had the strongest antioxidant ability, followed by aerial parts, stems, flowers and, roots. As shown in Figure 2, with one exception (metal chelation), the leaves exhibited the best ability in the methods performed. This fact also was confirmed by correlation analysis. The contradictory results from metal chelating assays might be explained with the presence of non-phenolic chelators such as polysaccharides, peptides, and sulphates. This approach was observed in earlier studies [62,63].

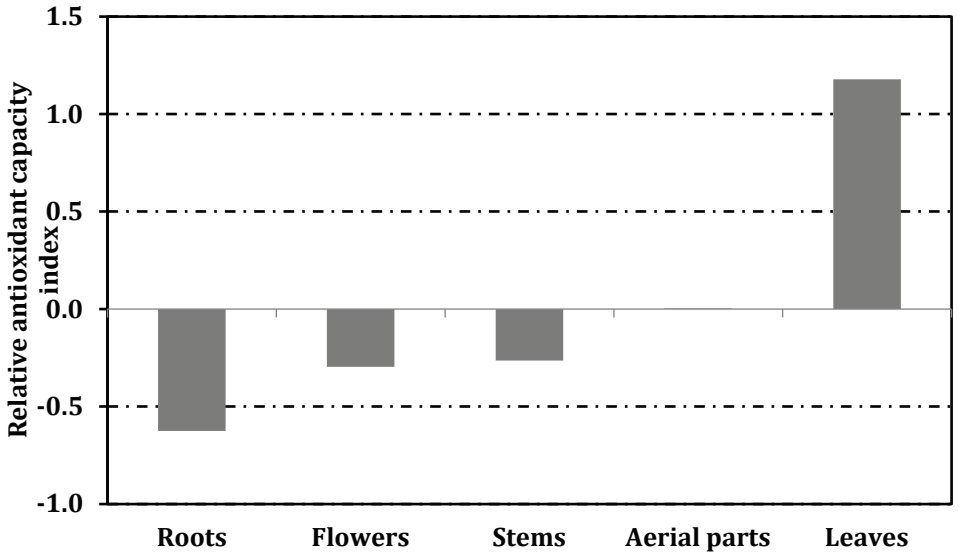

Figure 1. Relative antioxidant capacity index of different parts of *A. macrocephalus* subsp. *finitimus*.

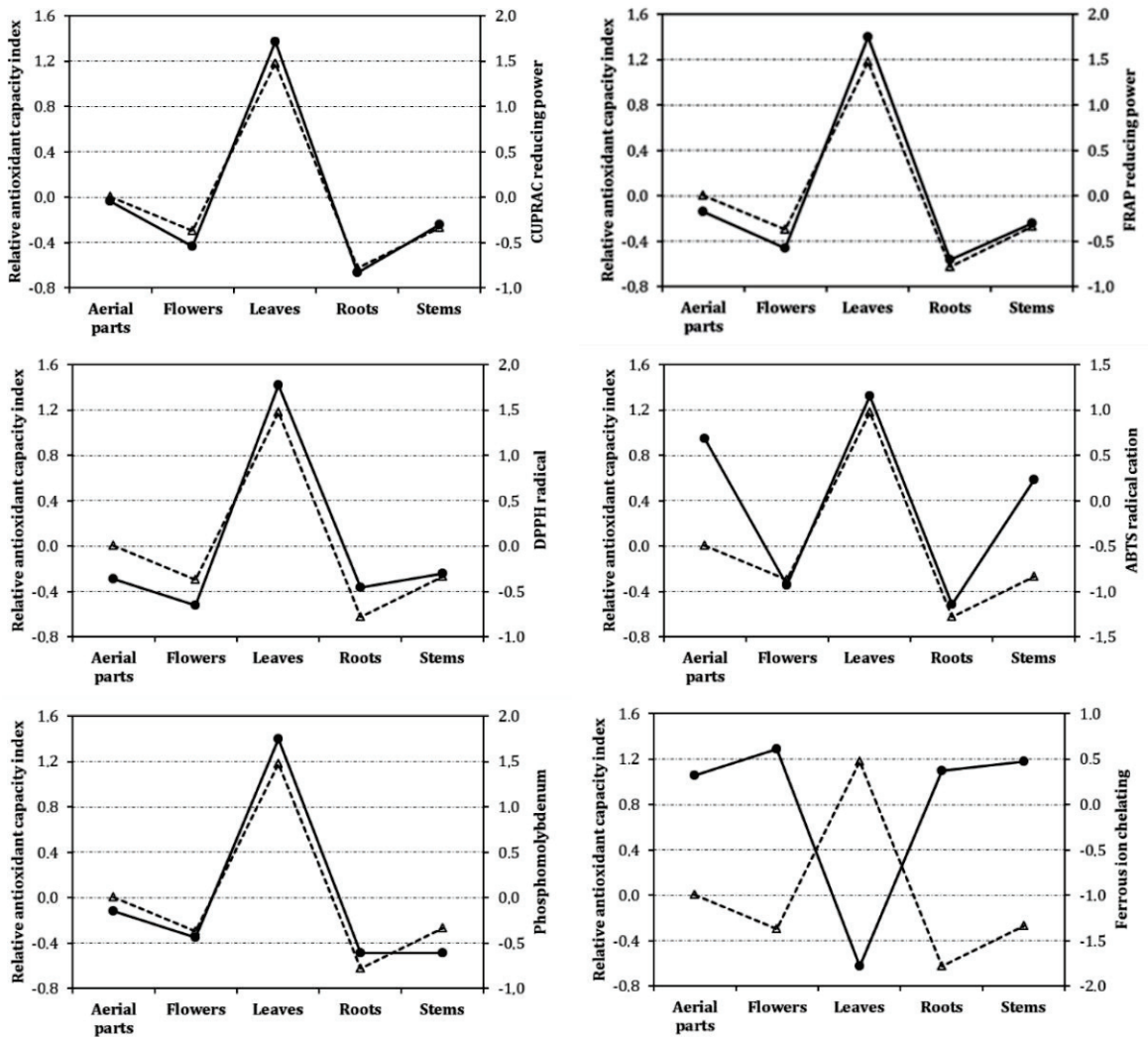

Figure 2. Relative antioxidant capacity index (dashed line with triangle) and antioxidant activity (solid line with circle) of each different part of *A. macrocephalus* subsp. *finitimus.*

3.3. Inhibitory Effects on Amylase and Tyrosinase

Enzyme inhibition theory is one of the most important strategies to combat global health problems including Alzheimer's disease and diabetes. In theory, some enzymes are targets to alleviate observed symptoms in the diseases [64]. For example, amylase is one of the main enzymes in the carbohydrate catabolism and it hydrolyzes α (1,4) glycosidic bonds in the starch. Thus, the inhibition of amylase can control the postprandial blood glucose level [65]. Additionally, tyrosinase is a key enzyme in the synthesis of melanin and its inhibition can reduce the symptoms of hyperpigmentation problems [66]. Thus, several compounds (acarbose for amylase and kojic acid for tyrosinase) have been developed as enzyme inhibitors in pharmaceutical industries. However, most of them have serious side effects such as gastrointestinal disorders and toxicity [67–69]. In this sense, natural substances prefer as enzyme inhibitors against synthetic ones.

Amylase and tyrosinase inhibition of *A. macrocephalus* subsp. *finitimus* extracts were investigated and the results are reported in Table 5. Similar to antioxidant assays results, the best inhibitory ability was detected in leaves extract (IC50: 3.36 mg/mL for amylase and 1.02 mg/mL for tyrosinase). In addition, the flowers exhibited the weakest inhibitory activities (IC_{50}: 4.94 mg/mL for amylase and 1.41 mg/mL for tyrosinase). The findings could be related with chemical profiles of the tested extracts and some compounds in extracts such as hyperoside [70,71], ferulic acid [72,73], and apigenin [74,75] have been reported as inhibitory agents in earlier studies. A moderate positive correlation was also observed

between total phenolic content and the enzyme inhibitory abilities Table 4. As far as we know, no information on the enzyme inhibitory effect of *A. macrocephalus* is present. Therefore, our results could provide new information on the biological activity poof for the genus *Astragalus*. At this point, *A. microcephalus* could be considered as a valuable source of natural enzyme inhibitors to combat global health problems including diabetes mellitus and skin disorders.

3.4. Principal Component Analysis

Unsupervised principal component analysis and hierarchical clustered analysis were applied to assess the connections between plant parts used on their biological activities. The outcomes are shown in Figure 3. With the percentage of variance of 79.1 and 9% respectively; the first two dimensions that represented a cumulative percentage of 88.1% of variance, seemed sufficient to cover the most information in the dataset. The main dominant biological activities of PC1 were FRAP, DPPH, CUPRAC, Ferrous ion chelating and phosphomolydbdenum while PC2 was dominated by alpha amylase inhibition Figure 3A. Regarding the loading plot, it can be seen that many biological activities were linked with each other Figure 3B. In fact, the greatest positive correlation occurred among tyrosinase and antioxidant properties. The existence of an interesting relationship between antioxidant defense systems and melanogenesis is well documented [76]. In fact, by reacting with toxic ROS result in the restriction of radical chain propagation, eventually preventing the skin from damage. Besides, the cytoprotective antioxidants can be increased by antioxidant molecules thanks to the nuclear accumulation of Nrf2, which is a main transcription factor for the oxidative stress regulation in human skin tissues such as melanocyte, keratinocytes, and dermal fibroblasts [76].

Further, it can be noted the involvement of polyphenols namely hyperoside, (−)-epicatechin, caffeic acid and 2,5 dihydroxybenzoic acid in these activities. Caffeic acid, an important members of hydroxycinnamic acid, (−)-epicatechin and 2,5 dihydroxybenzoic acid are reported to be a good antioxidant with an excellent tyrosinase inhibition properties [77–80]. In fact, the assays performed on the B16 melanoma cell line showed that caffeic acid can inhibit melanin production by suppressing casein kinase 2 induced phosphorylation of tyrosinase in dose dependent [81]. In addition, a flavanol glycoside, hyperoside is found to be a useful therapeutic agent in the vitiligo management and in the prevention of the oxidative stress induced by reactive oxygen species [82,83]. Regarding the ferrous ion chelating ability it might be predominantly related to the presence of syringic acid, 4-hydroxybenzoic acid, luteolin and eriodictyol.

Looking at the samples plot, a separation between the organs was achieved along PCs, with the leaves and flowers very distant from the three other organs (roots, aerial parts, and stem) (Figure 3C). Afterwards, the hierarchical analysis done on the basis of PCA result, brought out three clusters (Figure 3D). The results obtained in the current study, demonstrate that biological activities of plant differ dramatically from one organ to another. Among the analyzed organs of *A. macrocephanus*, leaves were found to be a promising source, enclosing biomolecules responsible for antioxidant properties and melanoma management ability. This is the result of the difference in quantity and quality of phytocompounds synthesizes in those organs. This quantitative and qualitative difference of phytocompounds is due to the anatomical and morphological structure as well as in several physiological processes that occur in the different organs [84].

Table 3. Antioxidant activities of standards and the methanol extracts from different parts of *A. macrocephalus* subsp. *finitimus* [x].

Assays	Aerial Parts	Flowers	Leaves	Roots	Stems	Trolox	EDTA
Effective concentration (EC_{50}: mg/mL)							
Phosphomolybdenum	2.52 ± 0.23 ^{c}	2.67 ± 0.08^{c}	1.81 ± 0.12 ^{b}	2.77 ± 0.03 ^{c}	2.77 ± 0.15 ^{c}	1.14 ± 0.02 ^{a}	-
DPPH radical	9.30 ± 0.20 ^{c}	14.20 ± 0.12^{e}	2.65 ± 0.05 ^{b}	10.54 ± 0.52 ^{d}	8.73 ± 0.22 ^{c}	0.26 ± 0.02 ^{a}	-
ABTS radical cation	1.66 ± 0.01 ^{b}	3.35 ± 0.01^{d}	1.45 ± 0.04 ^{b}	3.88 ± 0.11 ^{e}	1.93 ± 0.07 ^{c}	0.25 ± 0.02 ^{a}	-
CUPRAC reducing power	2.37 ± 0.06 ^{c}	3.62 ± 0.16^{d}	1.06 ± 0.02 ^{b}	5.28 ± 0.39 ^{e}	2.90 ± 0.12 ^{cd}	0.32 ± 0.03 ^{a}	-
FRAP reducing power	1.75 ± 0.02 ^{c}	2.46 ± 0.05^{d}	0.73 ± 0.01 ^{b}	2.83 ± 0.12 ^{e}	1.93 ± 0.08 ^{c}	0.12 ± 0.02^{a}	-
Ferrous ion chelating	1.08 ± 0.01 ^{b}	1.03 ± 0.01^{b}	1.65 ± 0.05 ^{c}	1.07 ± 0.01 ^{b}	1.05 ± 0.03 ^{b}	-	0.036 ± 0.004 ^{a}
Antioxidant activity							
Phosphomolybdenum (mmol TEs/g extract)	1.85 ± 0.17 ^{b}	1.73 ± 0.05^{b}	2.56 ± 0.17 ^{a}	1.67 ± 0.02 ^{b}	1.67 ± 0.09 ^{b}	-	-
DPPH radical (mg TEs/g extract)	24.97 ± 0.61 ^{bc}	15.42 ± 0.15^{d}	94.66 ± 1.97 ^{a}	21.75 ± 1.21 ^{c}	26.80 ± 0.76 ^{b}	-	-
ABTS radical cation (mg TEs/g extract)	161.59 ± 0.86 ^{b}	78.94 ± 0.01^{d}	185.65 ± 5.03 ^{a}	67.98 ± 2.01 ^{d}	138.54 ± 4.74 ^{c}	-	-
CUPRAC reducing power (mg TEs/g extract)	126.91 ± 3.50 ^{b}	78.76 ± 3.96^{d}	297.74 ± 4.90 ^{a}	50.23 ± 4.64 ^{e}	101.35 ± 4.66 ^{c}	-	-
FRAP reducing power (mg TEs/g extract)	59.81 ± 0.67 ^{b}	42.59 ± 0.89^{c}	142.69 ± 2.15 ^{a}	37.05 ± 1.63 ^{c}	54.37 ± 2.15 ^{b}	-	-
Ferrous ion chelating (mg EDTAEs/g extract)	66.81 ± 0.36 ^{a}	70.11 ± 0.51^{a}	43.41 ± 1.39 ^{b}	67.45 ± 0.18 ^{a}	68.59 ± 2.00 ^{a}	-	-

[x] Within each row, means sharing the different superscripts show comparison between the samples using Tukey's test at $p < 0.05$. EC_{50} (mg/mL), effective concentration at which the absorbance was 0.5 for reducing power and phosphomolybdenum assays and at which 50% of the DPPH and ABTS radicals were scavenged and the ferrous ion-ferrozine complex were inhibited. EDTA, ethylenediaminetetraacetic acid (disodium salt). "-", not determined. TEs and EDTAEs, trolox and ethylenediaminetetraacetic acid (disodium salt) equivalents, respectively.

Table 4. Correlations among phenolic compounds and assays [x].

Assays and Compounds	Phosphomolybdenum	DPPH	ABTS	CUPRAC	FRAP	Ferrous Ion Chelating	Tyrosinase	α-Amylase
DPPH	0.974 [y]							
ABTS	0.717	0.712						
CUPRAC	0.980 [y]	0.968 [y]	0.828					
FRAP	0.985 [y]	0.987 [y]	0.789	0.995 [y]				
Ferrous ion chelating	−0.983 [y]	−0.995 [y]	−0.678	−0.960 [y]	−0.980 [y]			
Tyrosinase	0.549	0.723	0.512	0.606	0.655	−0.671		
α-Amylase	0.497	0.525	0.528	0.477	0.491	−0.552	0.439	
Total flavonoid	0.792	0.657	0.471	0.754	0.725	−0.682	0.009	0.012
Total phenolic	0.992 [y]	0.992 [y]	0.727	0.985 [y]	0.995 [y]	−0.991 [y]	0.642	0.478
Hyperoside	0.982 [y]	0.987 [y]	0.794	0.995 [y]	0.999 [y]	−0.979 [y]	0.662	0.494

[x] Data show the Pearson Correlation Coefficients between the parameters. [y] Significant at $p < 0.01$.

Table 5. Enzyme inhibition activities of standards and the methanol extracts from different parts of *A. macrocephalus* subsp. *finitimus* [x].

Assays	Aerial Parts	Flowers	Leaves	Roots	Stems	Kojic Acid	Acarbose
Inhibition concentration (IC_{50}: mg/mL)							
Tyrosinase inhibition	1.33 ± 0.10 [cd]	1.41 ± 0.05 [d]	1.02 ± 0.02 [b]	1.18 ± 0.01 [bc]	1.07 ± 0.03 [b]	0.36 ± 0.04 [a]	-
α-Amylase inhibition	3.40 ± 0.02 [b]	4.94 ± 0.15 [d]	3.36 ± 0.18 [b]	3.50 ± 0.03 [b]	4.12 ± 0.22 [c]	-	1.24 ± 0.06 [a]
Enzyme inhibitory activities							
Tyrosinase inhibition (mg KAEs/g extracts)	270 ± 20 [cd]	255 ± 8 [d]	352 ± 7 [a]	304 ± 3 [bc]	336 ± 9 [ab]	-	-
α-Amylase inhibition (mg ACEs/g extracts)	357 ± 2 [a]	245 ± 8 [c]	362 ± 20 [a]	347 ± 3 [a]	294 ± 16 [b]	-	-

[x] Within each row, means sharing the different superscripts show comparison between the samples using Tukey's test at $p < 0.05$. IC_{50} (mg/mL), inhibition concentration at which 50% of the α-amylase and tyrosinase activities were inhibited. "-" not determined. ACEs and KAEs, acarbose and kojic acid equivalents, respectively.

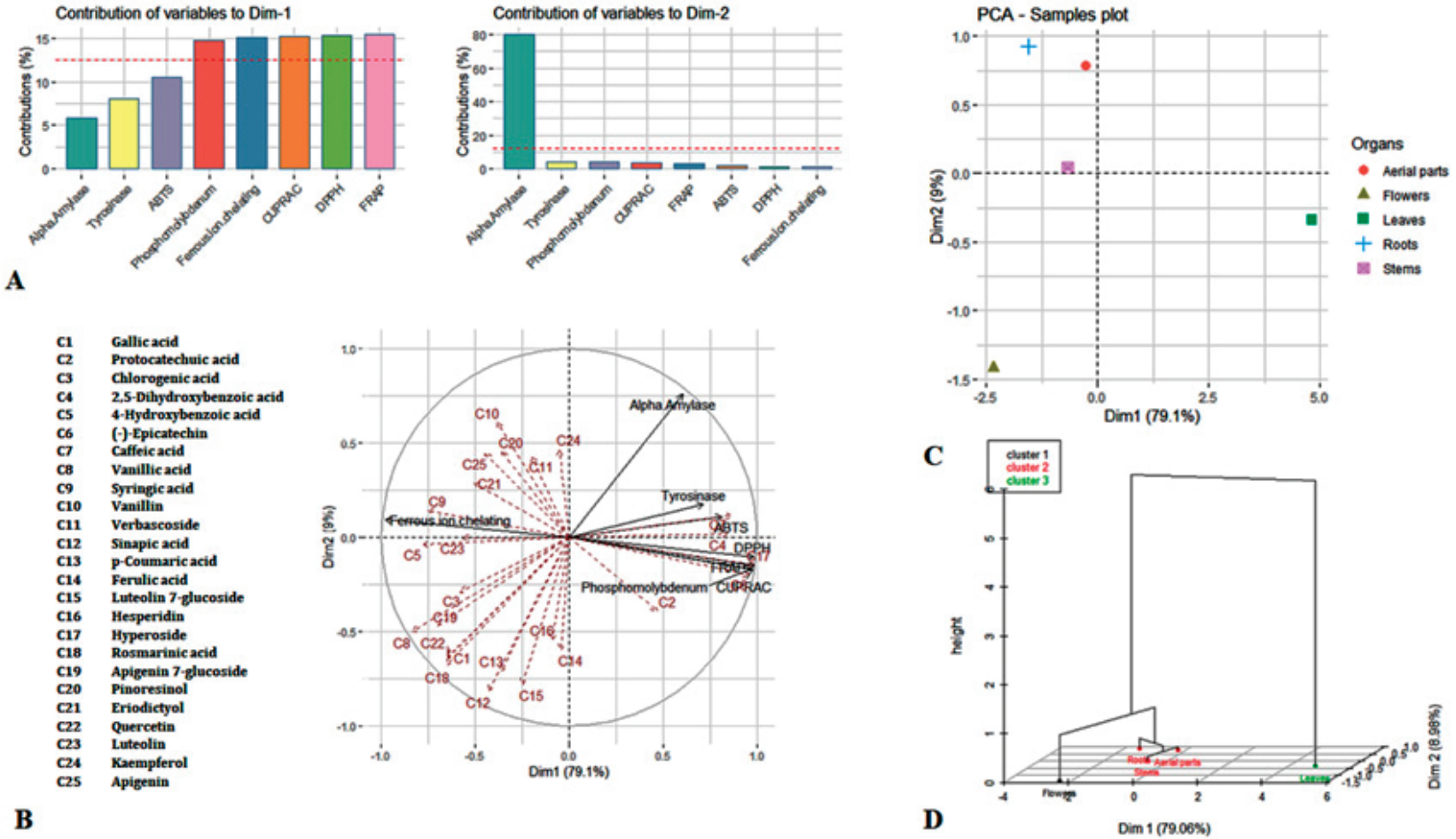

Figure 3. Principle Component Analysis (PCA) and hierarchical clustering analysis. (**A**): Loading plot. (**B**): Contribution of biological activities to each dimension of PCA. (**C**): Samples plot. (**D**): Hierarchical clustering on the fact.

4. Conclusions

Analysis of phenolic components, and biological potential using antioxidant and enzyme inhibitory assays of *A. macrocephalus* subsp. *finitimus* extracts were conducted for the first time. Twenty-four compounds were identified and quantified in the tested extracts. The levels of these compounds were dependent on the plant parts used. Hyperoside, apigenin, p-coumaric, and ferulic acids were dominant compounds in the extracts. In the connect with chemical profiles, different results were observed for each part in the biological activity assays. Except for metal chelating ability, the extract from leaves exhibited the best biological activities in the performed assays. To sum up, our observations suggest that *A. macrocephalus* subsp. *finitimus* could serve as a prominent source of bioactive agents to combat global health problems caused by oxidative stress. However, further studies are needed to understand the toxic profile, the type of enzyme inhibition and bioavailability of the tested extracts.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-7737/9/8/231/s1. In Section S.1 was given analytical methods applied for phenolic composition, antioxidant and enzyme inhibitory activities; Table S1: ESI–MS/MS Parameters and analytical characteristics for the Analysis of Target Analytes by MRM Negative and Positive Ionization Mode; Figure S1: LC-ESI-MS/MS chromatograms of the methanol extracts from aerial parts (**A**), flowers (**B**), leaves (**C**), roots (**D**), and stems (**E**) of A. macrocephalus subsp. finitimus.

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Review

Botanical Products in the Treatment and Control of Schistosomiasis: Recent Studies and Distribution of Active Plant Resources According to Affected Regions

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Abstract: Schistosomiasis, a parasitic disease caused by trematodes of the genus *Schistosoma*, is the second most prevalent parasitic disease in the world. It affects around 200 million people. Clinical treatment, prophylaxis, and prevention are performed in countries susceptible to schistosomiasis. In the pharmacological treatment for an acute form of schistosomiasis, the use of antiparasitics, mainly praziquantel, is more common. As an alternative way, prevention methods such as reducing the population of intermediate hosts (mollusks) with molluscicides are important in the control of this disease by interrupting the biological cycle of this etiological parasite. Despite the importance of pharmacological agents and molluscicides, they have side effects and environmental toxicity. In addition, they can lead to the development of resistance enhancing of parasites, and lead to the search for new and effective drugs, including resources of vegetal origin, which in turn, are abundant in the affected countries. Thus, the purpose of this review is to summarize recent studies on botanical products with potential for the control of schistosomiasis, including anti-Schistosoma and molluscicide activities. In addition, species and plant derivatives according to their origin or geographical importance indicating a possible utility of local resources for countries most affected by the disease are presented.

Keywords: antiparasitics; molluscicide; schistosomiasis; medicinal plants; Africa; Asia; Brazil

1. Introduction

Schistosomiasis is also commonly known as bilharziasis or snail fever. It is a parasitic disease caused by trematodes of the genus *Schistosoma* [1], which in turn are transmitted to humans through their intermediate hosts such as planorbids belonging to genus *Biomphalaria (S. mansoni)*, *Oncomelania* (*S. japonicum*), and *Bulinus* (*S. haematobium*) [2,3]. According to the World Health Organization (WHO), schistosomiasis is the second most prevalent parasitic disease in the world after malaria. It is associated with socioeconomic problems and water supply. It directly affects around 290 million people, with more than 700 million of them in risk areas. It is distributed in 78 countries, with major prevalence in South America, Sub-Saharan Africa, and Asia [4,5]. The worldwide burden associated with schistosomiasis has been projected to be at 2.6 million disability adjusted life years [6].

Main clinical manifestations of this disease include hepatomegaly, splenomegaly, periportal fibrosis, and appendicitis. An investigation for the different regions of Africa has revealed a high prevalence of infection [7]. In Mbita and the islands close to Lake Victoria, the prevalence of this disease in school children aged 5 to 19 years was 60.5%. In Lake Rweru, Rwanda, 21.1% of inhabitants were infected [8]. One study has been conducted in Nigeria to document the proportion of pregnant women affected by this disease. It was found that an astonishing 20.8% of women aged 15 to 42 years were infected by this parasite [9]. In areas where the disease is endemic, it is one of the main causes of pulmonary hypertension [10]. The prevalence of this disease in different African regions is summarized in Table 1.

Table 1. Summary of African regions where schistosomiasis strikes most.

Sub-Saharan African Region	Infected Individuals (Proportion)	Causal Agent	Source
Alamata district, Ethiopia	(73.9%)	*S. mansoni*	[7]
Nigeria	(56%)	*S. mansoni*, *S. mansoni* and *S. haematobium* combined infection	[11]
Sengerema district, nyamatongo ward, north-west Tanzania	School children aged 8–17 years (64.3%)	*S. mansoni*	[12]
Tono irrigation canal, north Ghana	Children aged 6–15 years (33.2%/19.8%)	*S. haematobium*/*S. mansoni*	[12]
Volta basin, Ghana	Adult male and female subjects (46.5%)	urinary schistosomiasis	[13]
Eastern cape province, South Africa	School-age students (73.3%)		[14]
School children, Mozambique	(47%/1%)	*S. haematobium*/*S. mansoni*	[15]
Zarima town, north-west Ethiopia	319 elementary school children (37.9%)	*S. mansoni*	[16]
South-west Cameroun	69.17%	*S. haematobium*	[16]

The transmission of schistosomiasis mainly depends on the presence of the infected person and faecal or urinary release of eggs from helminths into water environments containing the host mollusk, thus maintaining the life cycle of the parasite [4]. Globally, there are six species of schistosomes that can infect a human, including *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma haematobium* and, to a lesser extension, Schistosoma intercalatum, Schistosoma mekongi, and *Schistosoma guineesis* [17].

In the case of *S. mansoni* and *S. japonicum* infections, the acute form of this disease causes symptoms such as fever, myalgia, fatigue, malaise, dry cough, bloody mucus, diarrhea, diffuse abdominal pain, hepatosplenomegaly, eosinophilia, and the release of viable *Schistosoma* eggs in faeces, whereas dysuria, painful hematuria, urinary obstruction, vaginal discharge, or pain/bleeding after intercourse and the release of viable eggs in urine occurs in infections caused by *S. haematobium*. The chronic stage is caused by an egg deposition by individuals of the genus *Schistosoma* and reactions of the host's immune system. The fundamental characteristic of this form in *S. mansoni* and *S. japonicum* infections is the development of portal hypertension, leading to splenomegaly, hepatosplenic, and hepatointestinal forms. Varicose veins of the esophagus, spleen enlargement, hepatic cirrhosis, and urinary, intestinal, hepatic, and ectopic forms of the disease are other symptoms that may appear at this stage of the disease. In the infections caused by *S. haematobium*, the chronic stage is initiated by lodged eggs in the urogenital system, causing granulomatous host response and subsequent tissue inflammation [18].

Another form of the disease is neuroeschistosomiasis, a more frequent and disabling ectopic form compromising the central nervous system. Its diagnosis is based on epidemiological, clinical, and laboratory data. In some countries such as Brazil, this form of the disease has shown a considerable increase in the last two decades [19].

Clinical treatment, prophylaxis, and prevention are usually performed in countries susceptible to schistosomiasis. In the pharmacological treatment of acute schistosomiasis, the use of oxamniquine

and praziquantel, often associated, is common. Prevention methods such as reducing the population of intermediate hosts (mollusks) are also important in the control of the disease since they can interrupt the biological cycle of the etiological parasite [20,21].

2. Genus *Schistosoma* and Its Biological Cycle Importance in Schistosomiasis

The biological cycle of the parasite consists of two phases: The phase of the definitive host (vertebrate/man) and the phase of the intermediate host (mollusk). There are two larva passages of free life in the aquatic environment that alternate with parasitic phases. Adult worms live in blood vessels. In the case of *S. mansoni* and *S. japonicum*, they can attach to the intestine or the liver of the vertebrate host. Egg laying occurs in intestinal capillary vessels where they are directed into the intestinal lumen and exit into feces. Upon contact with water, the eggs will swell, hatch, and release ciliated larvae (miracidia) that can penetrate soft parts and develop the intermediate cycle upon finding snails. They can then generate sporocysts and cercariae later. Cercariae are the second parasite free life form. Finally, when cercariae find the skin of a vertebrate host, they become schistosomula and finally migrate to the liver where they become adults, completing the life cycle [22].

In liver infections, hepatic granuloma and periportal liver fibrosis are the most important pathogenic events in schistosomiasis and are mediated by several lymphocyte subpopulations, inducing inflammatory and fibrotic response around eggs housed in different tissues. At the time of oviposition, about 60% of eggs will reach the intestinal lumen. The rest will be destroyed in capillaries of the intestinal mucosa. Some eggs will remain there, while others will be carried by the mesenteric circulation to the liver where they will reside in hepatic sinusoids. The release of soluble antigens from eggs can induce the mobilization of macrophages, eosinophils, lymphocytes, and plasma cells. Macrophages are placed in contact with the egg, forming syncytial multinucleated masses. Some will differentiate into fibroblasts with an extensive production of collagen. Moreover, by migrating to the lungs and liver, schistosomula can cause arteriolitis, arteritis, and necrosis in addition to acute hepatitis with infiltration of neutrophils, lymphocytes, and eosinophils [23,24]. In general, the formation of granulomas in disparate organs and tissues explains manifestations of the disease, including portal hypertension, the formation of pseudotumors, neurological dysfunctions, and pulmonary vascular lesions. Another pathophysiological mechanism of relevance is the occurrence of an antigen-antibody reaction that may occur at high levels with the formation of circulating immunocomplexes that can be deposited in renal vessels, causing schistosomal nephropathy [24].

On the other hand, adult worms of *S. haematobium* species live within the urogenital venules, where they digest erythrocytes. The bladder, lower ureters, urethra, seminal vesicles, cervix, uterus, and vagina are most commonly affected. Unlike other schistosomes that live within the mesenteric venules and release their eggs into the host's intestines, *S. haematobium* releases its eggs into the urinary tract. The eggs can be eliminated by the urine or remain installed in the urogenital mucosa, causing polyps, nodules, and "sandy patches", a calcified schistosome ova within atrophied mucosa that seems like sand in cystocopy and colposcopy. Moreover, the progression of the urogenital infection can lead to fibrosis and calcification of the bladder wall, causing obstruction, bacteriuria, and bladder cancer [4].

The pathogenesis of schistosomiasis is derived from the host-parasite interaction. The strain, the evolutionary phase, the intensity, and the number of infections of *Schistosoma* are all important factors in its associated pathology [25]. On the other hand, the host organism response can vary according to genomic constitution, the predominantly injured organ, recidivate, food pattern, ethnicity, pharmacological treatment, associated infections, in utero sensitization and, above all, the immune profile before, during, and after infection. From the first 12 h after penetration of cercariae, an important dermal and subdermal inflammatory reaction is observed. This inflammatory reaction is predominantly caused by mononuclear and polymorphonuclear cells, leading to symptoms such as a pruritic maculopapular rash [25,26].

3. The Use of Drugs in the Treatment of Schistosomiasis

Pharmacotherapy is the most effective method for reducing the number of infection cases of schistosomiasis. In the last four decades, access to more effective drugs has reduced the prevalence and morbidity of this disease in various countries [4,27]. In the therapeutic treatment for an acute form of schistosomiasis, the use of prednisone, a corticosteroid, or its association with oxamniquine or praziquantel, is recommended. For the treatment of chronic disease without advanced lesions, praziquantel and oxamniquine are mostly indicated. In patients with involvement of the spinal cord (schistosomal myelopathy), the use of schistosomicides and steroids has been shown to be effective in most cases. At this rate, corticosteroids should be maintained for several months after clinical improvement and they should be withdrawn slowly. Such combination is also the therapy of choice for individuals with an advanced hepatosplenic form presenting portal or pulmonary hypertension who may develop hepatitis or pneumonitis due to the embolism of dead worms after treatment [28].

Mechanisms of action of the two main antiparasitics (praziquantel and oxamniquine) are very different. Praziquantel probably interferes with the muscular activity of the parasite, causing paralysis and preventing its binding with the host tissue. It also causes electrolyte imbalance and leads to destruction of the schistosome [29]. Oxamniquine acts by its anticholinergic effect. It can irreversibly inhibit enzymes that synthesize nucleic acids [30,31].

Despite the historical use of these schistosomicides, side effects such as metallic taste in the mouth, abdominal pain, diarrhea, asthenia, headache, dizziness, decreased therapeutic efficacy, and resistance have been reported [28]. Furthermore, the use of corticosteroids can culminate in the development of several side effects of this pharmacological class, such as Cushing's syndrome, metabolic dysregulation, and immunosuppression [32]. Other drugs used for this purpose also have many side effects with broad parasitic resistance and/or poor efficacy and some of them have been discontinued, such as metrifonate, an organophosphate with good efficacy against *S. haematobium*, but it caused abdominal pain, nausea, vomiting, diarrhea, headache, and vertigo [33]. On the other hand, artemisinine derivatives useful in the treatment of schistosomiasis and other parasitic diseases such as fasciolosis and triphostomy have been gradually neglected due to the increasing resistance in individuals co-infected with the malaria parasite [34], although these drugs can act synergistically with the heme group, generating free radicals that are toxic to the schistosomulae and are highly active against juvenile worms, whereas praziquantel is active against adult forms [35].

4. Biological Control of Intermediate Hosts as an Alternative Way

The control of the malacological population is an alternative way to prevent parasitic diseases that have mollusks in the life cycle of the parasite. The practice of combating natural breeding sites of intermediate hosts through the use of molluscicides has been one of alternative ways to decrease the incidence of some diseases such as schistosomiasis. However, most of these molluscicides have disadvantages such as damage to the ecosystem. Thus, there has been increasing interest in searching for new molluscicides from natural products that are less harmful to the environment [36].

Clinical treatment, prophylaxis, and prevention are performed in countries susceptible to schistosomiasis. In many cases, prevention methods such as reducing *Schistosoma* intermediate hosts (*Biomphalaria* and *Oncomelania*, for example) are important in controlling the disease, when combined with other strategies, such as the improvement of water sanitation, education, and access to clean water. However, vectors have developed resistance to chemical substances that are commonly used to inhibit the development and propagation of snails, leading to the search for new drugs and substances to be used in snail control [20].

Metallic salts such as copper sulphate were the first molluscicidal agents used. However, over time, they caused serious ecological imbalance since they limited the growth of algae that served as food for fish [3]. With the development of new molluscicides such as nicotinanilide, organotin, dibromo-nitrobenzene, sodium pentachlorophenate, tritylmorpholine, acetamide, and niclosamide, the environmental imbalance problem has become less drastic [37]. However, resistance in mollusks,

residual toxicity, and low selectivity of these agents continue to be important reasons for the search and development of more selective, safe, and effective molluscicides [36].

Niclosamide is a drug of choice for controlling mollusks involved in the schistosomiasis cycle. It is also used as anthelmintic in humans [38]. This substance acts by inhibiting the anaerobic phosphorylation of adenosine diphosphate (ADP) by mollusk mitochondria, thus blocking the process of obtaining energy dependent on the fixation of CO_2 [39]. In addition to the problems mentioned above, niclosamide is costly and easily degraded under sunlight [40].

In recent years, the aim to control and/or prevent schistosomiasis has been listed as a top priority in the agenda of the government, pharmaceutical companies, and international agencies triggered by initiatives derived from the World Health Agency resolutions [41]. The WHO program targets deworming of at least 75% of school children. However, only 28% of this figure was met in 2015 [42] mainly due to the scarce access to praziquantel and prophylactic chemotherapy. In addition, being the sole drug marketed for schistosomiasis therapy, there are some evidence points that praziquantel might be inefficacious against distinct developing strains of the parasite [43–45]. Other instances have demonstrated that intensive use of praziquantel can result in declined cure rates, higher resistance, and ultimately treatment failure [46]. The rising cost of praziquantel has also contributed to less successful prophylactic programs since most prevention programs depend on the availability of charitable funding organizations. In addition, in many countries, the control of schistosomiasis is not integral of the national budget, meaning that resources are limited to overcome the morbidity and mortality associated with the disease [41,42,47]. Given the limitation in available resources and the fact that there is no available vaccine for this disease, there is a dire need to develop alternative medicines that are both cost-effective and treatment-effective for this disease [48,49]. In this advent, natural products present an interesting opportunity toward the development of novel pharmacological agents to triumph over this disease.

The prevalence of schistosomiasis is largely dependent on the intermediate host ecology. Controlling the intermediate host undeniably can control the disease [50]. In this advent, plants with proven molluscicide activities are cheap and environmentally friendly for the control of parasite. Since the past century, several studies have focused on molluscicidal activities of endemic plants from the most affected regions in the world. Plant candidates include the following: *Apodytes dimidiata* E.Mey.ex Arn., *Ambrosia maritima* L., *Anacardium occidentale* L., *Croton macrostachys* L. Hochst.ex A.Rich., *Phytolacca dodecandra* L'Herit, *Swartzia madagascariensis* Desv., *Phytolacca dodecandra* L'Hér., *Sapindus Saponaria* L., *Swartzia madagascariensis* Desv., *Berkheya speciosa* (DC.) O.Hoffm., *Balanites maughamii* Sprague, *Warburgia salutaris* (G.Bertol.) Chiov., *Combretum imberbe* Wawra, *Combretum molle* R.Br. ex G.Don, *Euclea natalensis* A.DC., *Apodytes dimidiata* E.Mey. ex Arn., *Gardenia thunbergia* Thunb., and *Solanum nodiflorum* Jacq. [51–55].

On the other hand, several plants have the potential to interfere with the life cycle of worms, thereby impeding their growth, locomotion, and ability to lay eggs. A plethora of such plants have been highlighted by the scientific community, including *Jatropha elliptica* (Pohl) Oken, *Asparagus stipularis* Forssk, *Sanguinaria canadensis* L., *Curcuma longa* L., *Plectranthus neochilus* Schltr., *Hemerocallis fulva* (L.) L., *Schefflera vinosa* (Cham. and Schltdl.) Frodin and Fiaschi, *Cleome droserifolia* (Forssk.) Delile, *Clerodendrum umbellatum* Poir., *Artemisia annua* L., *Baccharis trimera* (Less.) DC., *Persea americana* Mill., *Allium sativum* L. [56], *Abrus precatorius* L., *Acacia karroo* Hayne, *Maytenus senegalensis* (Lam.) Exell, *Peltophorum africanum* Sond., and *Ziziphus mucronata* Willd. [57]

5. Recent Studies on Natural Resources of Affected Regions as an Alternative in the Treatment and Control of Schistosomiasis

Within the context mentioned above, the search for new and effective drugs with fewer side effects is necessary. Hence, the active agents of vegetal origin that are abundant in number and diversity in many affected countries could be a promising alternative. According to Ali (2011), several plant species have been used in different regions of the world for the treatment of parasitic diseases, including

Allium sativum, *Chenopodium ambrosoides*, *Curcubita pepo*, *Olea europaea*, *Mentha crispa*, *Citrus reticulata*, and phytopharmaceuticals such as berberine and lapachol [33,58]. However, an increasing number of studies have demonstrated the importance of endemic plant species and their derivatives with a specific action against the genus *Schistosoma* or against one of their intermediate hosts. Such endemic plant species and its derivatives can serve as prophylactic and/or curative agents in countries where they are found. The objective of this review is to summarize recent studies (since 2010) on endemic species, especially species from the most affected countries. In the following sections, we describe the most promising plant derivatives according to their native region.

5.1. Brazil

Brazil is one of the most affected countries by schistosomiasis, presenting 12,009 new cases in 2016 and about 1.5 million people are at risk of contracting this disease. *Schistosoma mansoni*, the main etiologic agent of this disease in Brazil, can be transmitted to the population through three species of mollusks: *Biomphalaria glabrata*, *B. straminea*, and *B. tenagophila* [59]. This country has a vast area of equatorial, tropical, and subtropical climate, so that it is suitable for the development of different strains of snails, as well as the parasite's cycle. On the other hand, the country also has the greatest biodiversity on the planet, which potentially has a large number of plant resources to be researched in the combat against schistosomiasis [60].

In 2010, Parreira et al. [61] described the activity of essential oil (10, 50, and 100 μg/mL) from leaves of *Baccharis dracunculifolia*, a species used by folk medicine, against *Schistosoma mansoni* adult worms. After the treatment, worms showed a significant decrease in their motor activities. In addition, most pairs of coupled adult worms were separated into individual males and females. Furthermore, adult worms of *S. mansoni* showed integumentary alterations after treatment with the essential oil. Their study also demonstrated that the essential oil had no toxicity against VERO cells. Furthermore, it showed that nerolidol and spathulenol were major substances in the essential oil [61]. In the same year, Magalhães et al. [62]. showed that the action of fluoroglucinol derivatives isolated from species from genus *Dryopteris* is known to have a global distribution against *S. mansoni.* Aspidin at 25 to 100 μM, flavaspidic acid at 50 and 100 μM, methylene-bisaspidinol at 100 μM, and desaspidin at 25 to 100 μM were the most active derivatives, causing death of adult worms. These fluoroglucionols at 100 μM also inhibited egg development. Furthermore, the authors suggested that schistosomicidal effects of phloroglucinols derivatives might be related to the inhibition of oxidative phosphorylation pathway in *S. mansoni* [62].

Ageratum conyzoides L. (Asteraceae), an annual aromatic weed from Southeastern Brazil, can produce leaf essential oil rich in Precocene I and (*E*)-caryophyllene [63]. The synergic effect of main compounds against adult worms of *S. mansoni* has been reported. *Plectranthus neochilus*, another Brazilian southeastern plant, has also been evaluated for its activity against *S. mansoni*. The essential oil of its leaves at a concentration of 100 ppm caused 100% mortality in a period of 24 h. Furthermore, it caused separation of coupled pairs, decrease of motor activity, and tegumental alterations. The main substances of its essential oil were β-caryophyllene, α-thujene, and α-pinene [63]. In the same year, another group of researchers also demonstrated the antiparasitic activity of cramoll-1,4-lectine isolated from seeds of *Cratylia mollis*, an endemic species from the Brazilian northeast [64]. Treatment with this substance at 50 mg/kg for 40 days or at 7 mg/kg for seven days reduced egg excretion (79% or 80%), adult worm recovery (71% or 79%), and liver granulomas (40% or 73.5%) caused by *S. mansoni* in infected mice [64].

In vitro activity of piplartine, an amide isolated from inflorescences of *Piper tuberculatum* found in Brazil, has been reported [65]. Piplartine at a concentration of 15.81 μM reduced the motor activity of worms of *S. mansoni* and caused their death within 24 h. In addition, this substance induced morphological changes on the tegument of adult worms. A quantitative analysis revealed an extensive tegumental destruction, represented by the number of damaged tubercles. In addition, this amide was not toxic to the VERO cells when it was used at concentrations up to three times higher than the one

showing schistosomicidal effects (31.51 μM) [65]. Another study demonstrated that imidazole alkaloid epiisopiloturine had an anti-*S. mansoni* activity. This substance was found in leaves of *Pilocarpus microphyllus*, a native plant species of Amazonia and Brazilian savannah. This alkaloid has been shown to be active against parasites of different developmental stages, including adults, schistosomulas, and eggs. Epiisopiloturine at a concentration of 300 μg/mL caused the death of all schistosomula within 120 h. Extensive tegumental alterations and death were observed when adult schistosomes were exposed to 150 μg/mL of epiisopiloturine. At the highest sub-lethal dose (100 μg/mL), a 100% reduction in egg laying of paired adult worms was observed. Furthermore, it exhibited no cytotoxicity to mammalian cells [66].

Oliveira et al. [67] have investigated the activity of *Baccharis trimera* against *S. mansoni*. This species is commonly used as a phytotherapic agent in Brazilian traditional medicine due to its several pharmacologic activities. They found a significant decline in the motility of worms with a mortality rate of 100% at 30 h after exposition to the leaf essential oil at a concentration of 130 μg/mL. Male worms were more susceptible, producing a dose-response effect within a shorter exposition period than female worms. The essential oil of *B. trimera* also induced a peeling on the tegument surface as well as destruction of tubercles and spines, resulting in smooth areas on the body surface. The essential oil also caused tegument destruction in female worms. In addition, it caused destruction of oral and acetabular suckers. Moreover, the cytocidal effect was only observed at the highest concentration (250 μg/mL), indicating a low cytotoxicity of this essential oil [67].

Miranda et al. [68] have investigated the antiparasitic action of steroidal alkaloids from *Solanum lypocarpum*, a Brazilian medicinal plant known as "wolf fruit". In vitro schistosomicidal activities isolated from steroidal alkaloids were evaluated against adult worms. The alkaloidic extract (20, 32, and 50 μg/mL), solasonine (50 μM), solamargine (32 and 50 μM), and equimolar mixture of glycoalkaloids (20, 32, and 50 μM) caused the separation of all coupled worms and extensive disruption on their teguments such as sloughing. It also caused death within 24 h of incubation. In addition, the alkaloidic extract (10 and 15 μg/mL), solasonine (50 μM), solamargine (10, 15, and 20 μM), and equimolar mixtures of glycoalkaloids (10 and 15 μM) reduced the development of eggs produced by adult worms. There was a synergistic effect between solamargine and solasonine [68].

In 2014, Brazilian researchers evaluated the molluscicidal activity of *Schinopsis brasiliensis* against *Biomphalaria glabrata*. Extracts in chloroform and ethyl acetate from the stem bark caused mortality of *B. glabrata*, with IC_{90} values of 68 and 73 μg/mL, respectively [59]. In 2018, Faria et al. investigated effects of plants on the control of *B. glabrata*. *Manilkara subsericea*, an endemic plant to the Brazilian sandbanks of Rio de Janeiro State, has a wide range of biological activities. At a concentration of 250 ppm, the *M. subsericea* leaf crude extract and ethyl acetate fraction induced 80 ± 4.13% and 86.66 ± 4.59% mortalities of adult snails after 96 h of exposure. LD_{50} values were 118.7 ± 1.62 and 23.41 ± 1.15 ppm, respectively. Substances isolated from *M. subsericea* were also found to be active in controlling *B. glabrata*. Treatment with quercetin, myricetin, and ursolic acid at a concentration of 100 ppm for 96 h induced mortalities of 100%, 80%, and 53.33%, respectively [69]. One year later, another study reported the activity of a Brazilian sandbank species against *B. glabrata* [70]. Nanoemulsified essential oil from leaves of *Xylopia ochrantha*, an Annonaceae plant, showed activity against three species of *Biomphalaria*: *B. glabrata*, *B. tenagophila*, and *B. straminea*. The similar action was observed on mollusks of different ages and its oviposition. Treatment with this essential oil at a concentration of 78 ppm for 24 h caused 100% mortality of all adult species [70]. Table 2 shows recent studies with Brazilian plants with antischistosoma or molluscicide activity.

Table 2. Extracts, oils, and substances from the Brazilian plant origin with antischistosoma or molluscicide activities.

Plant Source	Extract/Oil/Substance	Biological Target	Ref.
Ageratum conyzoides L.	Leaf Essential Oil	*Schistosoma mansoni*	[71]
Baccharis dracunculifolia	Leaf Essential Oil	*Schistosoma mansoni*	[61]
Baccharis trimera	Leaf Essential Oil	*Schistosoma mansoni*	[67]
Cratylia mollis	Cramoll-1,4-lectine	*Schistosoma mansoni*	[64]
Dryopteris genus	Aspidin, Flavaspidic Acid, Methylene-bisaspidinol, Desaspidin	*Schistosoma mansoni*	[71]
Pilocarpus microphyllus	Epiisopiloturine	*Schistosoma mansoni*	[66]
Piper tuberculatum	Piplartine	*Schistosoma mansoni*	[65]
Plectranthus neochilus	Leaf Essential Oil	*Schistosoma mansoni*	[63]
Solanum lypocarpum	Fruit Alkaloidic Extract, Solasonine, Solamargine, Glycoalkaloid mixture	*Schistosoma mansoni*	[68]
Manilkara subsericea	Crude Ethanolic Extract, Ethyl acetate Extract, Quercetin, Myricetin, Ursolic Acid	*Biomphalaria glabrata*	[69]
Schinopsis brasiliensis	Stem bark chloroformic and ethyl acetate extracts	*Biomphalaria glabrata*	[72]
Xylopia ochrantha Mart.	Leaf Essential Oil	*Biomphalaria glabrata, B. straminea* and *B. tenagophila*	[70]

5.2. Africa

In Africa, schistosomiasis is endemic in rural and coastal regions. Its geographical conditions are suitable for infestation of this parasite due to multiple creeks, creeklets, lakes, ponds, rivers, and almost stagnant water sources [73]. Being a neglected tropical disease, schistosomiasis strikes mostly poor, marginalized communities where people are in contact with natural water sources, posing an enormous societal, healthcare, and economic burden, mostly in Sub-Saharan Africa (SSA) [74,75]. Furthermore, this disease has a profound adverse effect on maternity, the development of children, and agricultural output. Schistosomiasis remains one of the prime factors contributing to poverty among 500 million SSA residents. It has been estimated that around 120 million individuals were present with schistosomiasis-related symptoms in Sub-Saharan Africa. This constitutes around 85% of the Sub-Saharan Africa population which represents 13% of the world's populace [76]. In addition, 20 million individuals from the same region undergo an incredible torment due to chronic manifestations of this disease which ranks the second right after hookworm infection in SSA. Among African countries with the highest prevalence of schistosomiasis, Nigeria has the highest number (29 million) of cases, followed by the United Republic of Tanzania, Ghana, and the democratic republic of Congo with 19 and 15 million cases each [17,34]. Nonetheless, it is believed that most cases of the disease are not reported. The true incidence of this disease might be 400–600 million cases worldwide [34].

5.2.1. Plants Traditionally Used against Schistosomiasis

A plethora of plant species have been employed traditionally against schistosomiasis in SSA. They have been recorded in both early and recent ethnobotanical surveys conducted in Africa. These plant species include *Abrus precatorius* L. subsp. africanus Verdc., *Afzelia quanzensis* Welw., *Antidesma venosum* E. Mey. ex Tul., *Boswellia carteri* Birdw., *Berkheya speciosa* (DC.) O.Hoffm., *Cassia abbreviata* Oliver subsp., *Cissampelos murconata* A. Rich. *Euclea divinorum* Hiern., *Euclea natalensis* A. DC., *Faurea saligna* Harv., *Macaranga kilimandscharica* Pax., *Maytenus senegalensis* (Lam.) Excell., *Mondia whitei* Skeels, *Ocimum americanum* L., *Ocimum canum* Sims, *Ormocarpum trichocarpum* (Taub.) Engl., *Pterocarpus angolensis* DC., *Protasparagus buchananii* (Baker) Oberm., *Rhus gueinzii* Sond, *Rumex lanceolatus* Thunb., *Rumex nepalensis* Spreng., *Sclerocarya birrea* (A. Rich.) Hochst. subsp. caffra, *Tephrosia macropoda* Harv.,

Terminalia phanerophlebia Engl. and Diels, *Vernonia amygdalina*, *Ximenia americana* L. var. americana, and *Ximenia caffra* Sond [77–81].

Among the plant species traditionally employed against *Schistosoma mansoni*, only a few have been subjected to scientific screening (Table 3). Principally, in vitro assays have been performed on these plant species and the most potent result was retrieved for the root extract of *Abrus precatorius* L. subsp. africanus Verdc. which nonetheless deserved to be evaluated further in vivo and in randomized clinical trials.

Table 3. In vitro studies of traditionally employed plant species against *Schistosoma mansoni.*

Plant Species	Part Used	Lethal Concentration (mg/mL) (t = 1 h)	Source
Abrus precatorius L. subsp. africanus Verdc.	Stem Root	1.50.6	[57]
Berkheya speciosa (DC.) O.Hoffm.	Aqueous plant extract	> 6.25	[82]
Euclea divinorum Hiern	Aqueous plant extract	50	[82]
Euclea natalensis A. DC.	Aqueous plant extract	> 3.13	[82]
Maytenus senegalensis (Lam.) Excell.	Leaves and stem Root Root and bark	25 25 2.5	[57]
Ocimum americanum hexane, *Ocimum americanum* water	Whole plant	Worm reduction in mice (68.7 and 63.4%) vs. praziquantel (75.2%)	[83]
Pterocarpus angolensis DC.	Leaves Stem Bark	102 33.8 51.3	[57]
Sclerocarya birrea (A. Rich.) Hochst. subsp. Caffra		> 25	[82]

t: Time; h: Hour.

5.2.2. Recent Scientific Studies on Molluscicide Activity

In 2010, Adetunji and Salawu [84] reported that *Terminalia catappa* and *Carica papaya* possessed molluscicide activity against *Bulinus globosus* and *Biomphalaria pfeifferi*, respectively, the main intermediate hosts of *S. haematobium* and *S. mansoni*. Despite the fact that both vegetal species are distributed in several tropical regions of the world, they also have good adaptation in Nigeria and affect mollusks and parasites of great importance in this country. In that study, an ethanolic extract of *T. catappa* leaf showed LC_{50} values of 864.1 and 2716.3 ppm against *B. pfeifferi* and *B. globosus*, respectively. However, an ethanolic extract of *C. papaya* leaf showed LC_{50} values of 1095.7 and 619.1 ppm against *B. pfeifferi* and *B. globosus*, respectively [84]. One year later, Nigerian scientists described activities of several extracts of leaves and fruits of *Blighia unijugata*, an endemic plant in tropical Africa, against *B. glabrata*. LC_{50} values of these extracts were found to be 7.60 and 13.00 μg/mL. Among various extracts, the ethyl acetate extract of its fruit was the most active one [57]. Part of these authors also evaluated the activity of *Zanha goluogensis* against the same mollusk. It was found that the ethanolic extract of its stem showed an LC_{50} value of 60 ppm [82].

In 2013, Angaye [85] investigated the activity of different extracts of *Jatropha curcas* against mollusks *Bulinus globosus*, *Bulinus rholfsi*, and *Biomphalaria pfeifferi*. It was found that the methanolic extract of its leaves was active against all species, showing LC_{50} values of 0.3 and 30 ppm against *Bulinus* and *B. pfeifferi*, respectively, while the crude ethanolic extract showed less activity (LC_{50} > 500 ppm) [85,86]. Previously, other authors have also reported activities of root and seed methanolic extracts against different species of *Bulinus* [87,88]. Two years later, Angaye et al. [89] reported activities of two typical mangrove species, *Avicennia germinans* and *Rizhophora mangle*, from Niger Delta. For the first species, the methanolic leaf extract was active against *Biomphalaria pfeifferi*, *Bulinus globosus*, and *Bulinus rholfsi*,

showing LC_{50} values of 175, 89.21 and 123.74 ppm, respectively, whereas the methanolic leaf extract of *R. mangle* showed LC_{50} values of 87.50 and 108.22 ppm for the two first mollusks and *B. rholfsi*, respectively [89]. Aqueous and ethanolic extracts of *Vernonia amygdalina* have been found to be toxic to adult *Biomphalaria pfeifferi*, showing an LC_{50} value of 338.8 ppm and an LC_{90} value of 614.8 ppm [90]. Interestingly, previous studies with this species have reported its antischistosomal activity [91–93] and its use in etnomedicine as a folkloric treatment against haematuria resulting from *Schistosoma haematobium* infection and stomach complaints due to infestation by *Schistosoma mansoni* [51]. Studies of African plant species and their molluscicidal activity are summarized in Table 4.

Table 4. Scientific studies of African plant species with molluscicidal activity.

Plant Species	Plant Part /Extract	Snail Species	LC_{50}	Source
Avicennia germinans (L.) L.	Leaf/Methanol	*Biomphalaria pfeifferi*	175	[89]
	Leaf/Methanol	*Bulinus globosus*	89.21	
	Leaf/Methanol	*Bulinus rholfsi*	123.74	
Blighia Unijugata Baker	fruit/ethyl acetate	*Biomphalaria glabrata*	7.60	[94]
	Pericarp/butanol		15	
	Pericarp/water		25	
Carica papaya L.	Leaf/ethanol	*Bulinus globosus*	619.10	[84]
		Biomphalaria pfeifferi	2716.30	
Croton floribundus Spreng	Leaf/Hexane	*Biomphalaria glabrata*	37.4	[95]
	Leaf/Methanol	*Biomphalaria glabrata*	14.8	
	Leaf/ethanol	*Biomphalaria glabrata*	4.2	
Euphorbia helioscopia L.	Leaf/cold water	*Bulinus wright*	80	[96]
	Leaf/hot water		96.6	
	Leaf/methanol		11.3	
	Leaf/chloroform		80.5	
	Leaf/acetone		8.9	
	Leaf/hexane		99	
Euphorbia schimperiana Scheele	Leaf/cold water		81.8	[96]
	Leaf/hot water		72.8	
	Leaf/ methanol		2.3	
	Leaf/ chloroform		3	
	Leaf/acetone		10.1	
Jatropha Curcas L.	Leaf/hexane	*Bulinus natalensis* and *Bulinus truncatus*	18	[87]
	Seed/methanol		0.25	
	Root/ethanol	*Bulinus truncatus* and *Bulinus natalensis*	60	[88]
	Leaves/methanol	*Bulinus globosus* and *Bulinus rholfsi*	0.3	[85]
	Leaves/crude extract	*Biomphalaria pfeifferi*	> 500	[86]
	Leaves/methanol	*Biomphalaria pfeifferi*	30	[86]
	Leaves/crude extract	*Biomphalaria pfeifferi*	> 500	[86]
	Seed/methanol	*Biomphalaria pfeifferi*	25	[87]
Jatropha glauca Vahl	Leaf/acetone	*Biomphalaria pfeifferi*	6.76	[96]
Rhizophora mangle L.	Leaf/chloroform	*Biomphalaria pfeifferi*	16.50	[96]
	Leaf/methanol	*Biomphalaria pfeifferi*	87.50	[89]
Rhizophora racemosa G. Mey.	Leaf/methanol	*Bulinus globosus*	87.50	[89]
	Leaf/methanol	*Bulinus rholfsi*	108.22	
	Leaf/methanol	*Biomphalaria pfeifferi*	150	
Terminalia catappa L.	Leaf/methanol	*Bulinus globosus*	125	[84]
	Leaf/methanol	*Bulinus rholfsi*	85.51	
	Leaf/ethanol	*Bulinus globosus*	1095.70	
Tetrapleura tetraptera (Schum. and Thonn.) Taub.	Leaf/ethanol	*Biomphalaria pfeifferi*	864.10	[97]
	Fruit/methanol	*Bulinus globosus*	1.33	
Zanha goluogensis Hiern	Stem/ethanol	*Biomphalaria glabrata*	60	[96]

5.2.3. In Vitro Investigation of Medicinal Plants against Schistosoma

One of the most extensive studies about plant schistosomicidal activity was conducted by Yousif et al. in 2011 [98]. These authors screened 281 Egyptian plants, of which 14 showed high activities against *S. mansoni* adult worms after treatment for 24 h: *Callistemon viminalis* (Soland. Ex Gaertn) Cheel,

C. rigidus R. Br., *C. speciosus* (Sims.) DC, *C. citrinus* Stapf. *Eucalyptus citriodora* Hook, *Eucalyptus rostrata* Dehnh. *Eugenia edulis* Vell, *E. javanica* Lam, *Melaleuca leucadendron* (L.) L. *M. stypheloides* Sm (all belong to Myrtaceae), *Cryptostegia grandiflora* R. Br. (Asclepiadaceae), *Zilla spinosa* (L.) Prantl (Cruciferae), *Ficus trijuja* L.(Moraceae), and *Fagonia mollis* Delile (Zygophylacae) (Table 5) [98]. Moreover, in Egypt, another study on asparagaline A, a triterpenoid isolated from roots of an African plant *Asparagus stipularis* Forss., demonstrated its activity against *S. mansoni* [99]. The administration of asparagalin A resulted in a retardation of worm growth and locomotion on the first day. It also showed a significant activity of egg-laying suppression at a 200 μg/mL concentration [99]. In this same year, Ramalhete et al. [100] reported that triterpenes isolated from the methanol extract of aerial parts of *Momordica balsamina* L. had a potent in vitro schistosomicidal potential (100% within 24 h). Balsaminol F and karavilagenin C showed LC_{50} values of 14.7 ± 1.5 and 28.9 ± 1.8 μM, respectively, after 24 h of incubation. Both compounds at 10–50 μM induced significant reductions in the motor activity of worms and significantly decreased egg production. Furthermore, at 10-100 μM, they were able to separate adult worm pairs into males and females after 24 h [100].

Table 5. Egyptian species with activity against *S. mansoni* by Yousif et al. [95].

Plant Species	Part	LC_{50} (ppm)	LC_{90} (ppm)
Myrtaceae			
Callistemon viminalis (Soland. Ex Gaertn) Cheel	Leaves	6.56	9.49
	Branches	1.49	2.26
C. rigidus R. Br.	Aerial Roots	1.89	3.80
C. speciosus (Sims.) DC	Leaves/Branches	1.80	5.50
C. citrinus Stapf	Leaves	1.89	3.80
	Branches	1.80	4.10
Eucalyptus citriodora Hook	Bark	5.95	6.90
	Branches	10.00	11.07
Eucalyptus rostrata Dehnh.	Branches	7.80	13.50
Eugenia edulis Vell	Leaves	5.93	10.08
E. javanica Lam	Branches	9.70	12.90
Melaleuca leucadendron (L.) L.	Leaves	1.90	2.50
	Branches	2.30	6.20
M. stypheloides Sm.	Leaves/Branches	4.80	8.70
Asclepiadaceae			
Cryptostegia grandiflora R. Br.	Branches	11.40	23.80
Cruciferae			
Zilla spinosa (L.) Prantl	Fruits	10.50	48.90
Moraceae			
Ficus trijuja L.	Branches	14.40	39.50
Zygophylacae			
Fagonia mollis Delile	Herb	3.70	22.40

In 2015, a study investigated the effect of an aqueous extract (1.25–40 mg/mL) of aerial parts of *Sida pilosa* Retz. (Malvaceae) and derived n-hexane, dichloromethane, ethyl acetate, and n-butanol fractions (0.25–8 mg/mL) against *Schistosoma mansoni*. Among these extracts, the n-butanol fraction was the most active one, showing an LC_{50} value of 1.25 mg/mL [45]. Two years later, Tekwu et al. [101] demonstrated that the stem bark and root extracts of *Rauwolfia vomitoria* at concentrations of 250–1000 μg/mL were active against *Schistosoma mansoni* worms after 120 h of incubation. In addition, the cytotoxicity (MTT) assay conducted on HepG2 and Chang liver cells demonstrated that these extracts were safe. They inhibited the proliferation of these cell lines with an $IC_{50} > 20$ μg/mL [101].

5.3. Asia

5.3.1. Prevalence of Schistosomiasis in Asia

In Asia, the major foci of human schistosomiasis infection caused by parasite *Schistosoma japonicum* are in China, the Philippines, and small pockets of Indonesia. To a lesser extent, along the Mekong river on borders of Cambodia and Laos People's Democratic Republic, the infection is caused by *S. mekongi* [102,103]. *S. japonicum* and *S. mekongi* are traditionally considered as zoonotic [104]. Specific intermediate snail hosts for *S. japonicum* and *S. mekongi* are *Oncomelania hupensis* and *Neotricula aperta*, respectively [102,105]. Approximately 600 million people are estimated to be at risk of infection in China. Approximately 0.3 million people are currently infected. In the Philippines, 6.7 million people live in endemic areas. Of these, 1.8 million people are considered to be directly exposed to infection through water contact activities [106,107]. Approximately 140,000 people are estimated to be at risk for *S. mekongi* infection (80,000 people in Cambodia and 60,000 in Lao PDR) [102,108].

In China, schistosomiasis japonica remains a major public health concern. It is listed as one of the top priorities in communicable disease control defined by the central government [109,110]. The major endemic foci are concentrated on marshland and lake regions of Southern China which cover a vast area of five provinces (Jiangsu, Anhui, Hubei, Jiangxi, and Hunan). Cases within the area account for 86% of the total number of people infected in China [103,111]. It should be noted that the schistosomiasis transmission period in China lasts only for five months annually over two distinct transmission periods, whereas in the Philippines it is throughout the year [103,112]. For *S. mekongi*, its transmission period is March to April, coinciding with the dry season when the water level is low and host snail populations reach their maximum [102]. In Japan, eradication of the disease was achieved through transmission control by environmental management (i.e., land reclamation to enhance agricultural production and cementing ditches used for rice irrigation) and social economic development [103,113]. China has used extensive, long-term, repeated praziquantel chemotherapy to control the morbidity and reduce the prevalence and intensity of the *S. japonicum* infection in the country over three decades. Although *S. japonicum* has not shown resistance to praziquantel yet [114], the emergence of drug resistance has been experimentally induced in the laboratory, proving that *S. japonicum* may develop resistance to praziquantel under drug selection pressure [115,116]. As the potential development of praziquantel resistance may pose a great threat to the elimination of *S. japonica* in China and other Asian countries, there is an urgent need to find antischistosomal agents, especially from natural sources that are generally safer.

5.3.2. Plants Traditionally Used against Schistosomiasis in Asian Countries

Ginkgo biloba is a Chinese-specific rare relict species with relatively high economic and medicinal values. Its sarcotesta is usually discarded. However, it contains high levels of ginkgolic acids. Ginkgolic acids are long-chain phenolic compounds that are derivatives of sumac acid. Reports have shown that ginkgolic acids possess biological activities, including anti-tumor, neuroprotective, anxiolytic, and antibacterial activities [117–120]. Considering such biological virtues of *G. biloba*, Li et al. [121] have evaluated the petroleum ether fraction of the ethanolic extract of fallen leaf of *G. biloba* (PFGB) against *O. hupensis*. This extract showed a high toxicity (100% mortality) after treatment at a concentration of 100 mg/L for 72 h. Out of five fractions of the ethanolic extract of *G. biloba* tested for molluscicidal activity, the petroleum ether fraction (PFGB) exerted the most pronounced activity in a time- and dose-dependent manner. Experimental results demonstrated that the LC_{50} value of PFGB was decreased from 72.38 mg/L at 24 h to 9.22 mg/L at 72 h. Upon assessment, it was found that the glycogen and total protein contents of the snail's tissues decreased at a rate parallel to molluscicidal activity. Therefore, abnormal energy metabolism might be a factor contributing to its molluscicidal activity [121].

Buddleja lindleyana is a medicinal plant distributed in Eastern China, including Jiangsu, Anhui, Jiangxi, and Hubei provinces. This plant has been traditionally used for treating various ailments such as rheumatism, cough, and blood stasis [122]. Chemical investigations of *B. lindleyana* have

revealed compounds such as phenethyl alcohol glycosides, phenylpropanoid phenolic glycosides, sesquiterpenes, diterpenes, triterpenes, flavonoids, and other constituents. One study has revealed that the N-butanol fraction of *B. lindleyana* (NFBL) is potent against the snail *O. hupensis* (LC_{50} = 39.1 ppm, LC_{90} = 59.3 ppm) [120]. Han et al. [123] have further reported that active components of *B. lindleyana* can induce remarkable decreases of activities of five key enzymes: Succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), cytochrome oxidase (CC0), cholinesterase (CHE), and nitric oxide synthase (NOS). These decreased activities of enzymes affected the supply of neurotransmitters (CHE and NOS) and energy supply (CCO, LDH, SDH), leading to a physiological function disorder or loss which ultimately resulted in the death of snails [123]. A further study has revealed that acacetin-7-rutinoside is the molluscicidal component of NFBL. In addition, acacetin-7-rutinoside at a concentration of 100 mg/L was found to be non-toxic to zebrafish, implying that this active compound might have applications as a potent molluscicide against *O. hupensis* [124].

Pulsatilla chinensis (Bunge) Regel (PRS) is a botanical with a long history in medical use in China. It displays "blood-cooling" and detoxification activities. Roots of *Pulsatilla chinensis* (Bunge) Regel have been widely used for treating various ailments such as intestinal amebiasis, malaria, trichomoniasis, bacterial infections, and malignant tumor [125]. A study carried by Chen et al. in 2012 showed that PRS displayed similar molluscicidal activity against *O. hupensis* (LC_{50} at 24 h: 0.48 mg/L) to niclosamide as a positive control (LC_{50} at 24 h: 0.16 mg/L). Effects of PRS on cholinesterase (CHE), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) activities in cephalopodium and liver of snails were assessed. It was found that there were significant alterations in CHE, ALP, and ALT activities in the cephalopodium and the liver of snails after exposure to 40% and 80% LC_{50} of PRS or NIC for 24 h. Furthermore, the zebra fish lethality test was performed to assess its toxicity to non-target aquatic species. Results showed that PRS which contained 15 compounds was less toxic to zebra fish than the control (niclosamide) [126]. In 2018, Kang et al. investigated whether hederacochiside C (HSC) isolated from roots of *P. chinensis* possessed antischistosomal effects and anti-inflammatory activities in *S. japonicum*-infected mice. Mice infected by schistosomula or adult worms by an intravenous injection were treated with different concentrations of HSC twice a day for five consecutive days. Experimental results demonstrated that the total worm burden, female worm burden, and egg burden in livers of mice treated with 400 mg/kg HSC were fewer than those in non-treated ones. Following the HSC treatment, murine immune responses were assessed by enzyme-linked immunosorbent assays (ELISA). Results showed that 200 mg/kg of HSC was sufficient to reduce the expression of IgG, tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-17 in comparison with those in the infected group, exhibiting remarkable immunomodulatory effects [127]. More recently, Kang et al. reported antischistosomal properties of hederacolchiside A1 (HSA) isolated from *Pulsatilla chinensis* against *S. japonicum* and *S. mansoni*. Its antischistosomal activity was higher than praziquantel and artesunate against one-day-old juvenile schistosome. In vivo assays confirmed that the HSA-mediated antischistosomal activity was partly due to morphological changes in the tegument system when juvenile schistosomes were exposed to HAS [127]. Extensive tegumental disruption such as sloughing and erosion was observed. The tegument of schistosome is a protective sheath that plays a crucial role in host-parasite interactions as well as in defense, uptake of nutrients, osmoregulation, and excretion and hence morphological changes in the tegument is critical for the survival of schistosome [56,128].

Reports have highlighted the molluscicidal activity of the leaf extract *from C. camphora* against *O. hupensis* [129,130]. Yang et al. [131] have investigated molluscicidal and larvicidal activities of leaf extracts of *C. camphora* growing in China against *O. hupensis* and *S. japonicium*. Gas chromatography coupled to mass spectrometry (GC-MS) was performed to identify bioactive components from the leaf extract of *C. camphora*. Results showed that Linalool-rich *C. camphora* leaf extracts had high molluscicidal effects against *O. hupensis* (LC_{50} = 0.25 mg/L) and cercaricidal activity against *S. japonicium* (LC_{50} = 0.07 mg/L) [131].

Xiao et al. [132] have evaluated schistosomicidal activities of flavonoids isolated from *Astragalus englerianus*, a traditional Chinese medicine plant. Among the isolated flavonoids, 2,2′,5′-trihydroxy-4-methoxychalcone and (3R)-sativan caused 100% mortality of worms within 12 h after treatment with a RPMI 1640 medium containing each drug (0.70 and 0.77 mM, respectively) [132].

Wan et al. [133] evaluated cercaricidal activities of *Allium sativum* (garlic) oil against *S. japonicum* larvae both in vitro and in vivo. Their findings revealed that exposure to garlic emulsions at concentrations of 10^{-6} (*v*/*v*) or higher for 30 min induced a 100% mortality of *S. japonicum* cercariae. The toxicity of the garlic oil against *S. japonicum* was determined by pre-treating mice with garlic emulsion on the shaved abdomen, followed by an *S. japonicum* cercariae challenge. The in vivo assay revealed that the pre-exposure treatment with $\geq 10^{-4}$ (*v*/*v*) garlic emulsions in mice caused a 100% inhibition of *S. japonicum* infection in mice, while the pre-treatment with 10^{-5} and 10^{-6} (*v*/*v*) emulsions achieved 20–40% inhibition of *S. japonicum* infection and 35.2% to 63.6% worm burden reduction, respectively [133]. Similarly, curcumin, a major polyphenol isolated from rhizomes of *Curcuma longa* L., a dietary spice widely used in Asian cuisine and in folk medicines worldwide, has been reported to be active in vitro against every life stage of *S. japonicum* [134]. Furthermore, curcumin was observed to exert an optimal activity against the adult stage without differential sensitivity between male and female worms. After 72 h of incubation with 5 mg/mL of curcumin, a decrease in the motor activity of these worms without tegumental alterations was observed by Ke et al. [134].

Macleaya cordata (Willd) R. Br. is a plant with high content of alkaloids. The molluscicidal effect of alkaloid components against snail *Oncomelania hupensis* was determined by Ke et al. [135]. Alkaloid AN2 was found to be the most toxic one against snail *O. hupensis*, showing 48 h LC_{50} and LC_{90} values of 6.35 and 121.23 mg/L, respectively. Responses of some critical enzymes to AN2, including activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP), malic dehydrogenase (MDH), aspartate transaminase (AST), and succinate dehydrogenase (SDH), in cephalopodium and liver were also detected. Results showed that AN2 significantly inhibited activities of MDH, SDH, and esterase isozyme. AN2 also significantly stimulated activities of ALT, ALP, and AST to increase at a low concentration (25 mg/L), while IT significantly inhibited activities of these enzymes at a high concentration (100 mg/L). These results indicate that AN2 not only can inhibit protein synthesis and respiratory chain oxidative phosphorylation, but also can cause hepatocellular injury and reduce the detoxification ability of the liver [132]. Based on the aforementioned facts and information summarized in Table 6, many Chinese herbal medicines are potential molluscicides. They can be used to control intermediate hosts of schistosome and are safer to use than their synthetic counterparts.

Table 6. Molluscicidal activities of medicinal plants from China against *Oncomelania hupensis*, the intermediate host of *Schistosomiasis japonica*.

Species Name	Plant Parts Used	Extraction Solvent	Activity (%Mortality/ LC or LD) Value	Active Compounds	References
Acorus gramineus	Rhizome, leaf	Ethanol	Exposure time = 72 h; Dose = 200 mg/L Rhizome: 75% mortality Leaf: 56.25% mortality		[136]
Buddleja lindleyana	Leaf	Ethanol	Ethanol: 100% mortality caused after 72 h exposure at a concentration of 200 mg/L	Acacetin-7-rutinoside: LC_{50} = 36. 12 mg/L (24 h)	[123,136]
		N-butanol for fractionations	N-butanol fractions: Exposure time = 72 h; Dose = 50 mg/L LC_{50} = 39.1 mg/L LC_{90} = 59.28 mg/L	LC_{50} = 3.26 mg/L (72 h)	[124]
Clerodendron cyrtophyllum	Branch, Leaf	Ethanol	Exposure time = 72 h; Dose = 200 mg/L Branch: 56.25% mortality Leaf: 65% mortality		[136]
Eupatorium adenophorum	leaf, roots and stems	Water	Leaf extract: 100% mortality caused after 82 h exposure with 0.27% (*w*/*v*) extract Roots extract: 56.7% mortality after 76 h with 0.86% (*w*/*v*) extract Stem extract: 40 7% mortality after 82 h with 0.86% (*w*/*v*) extract		[137]
Ginkgo biloba	sarcotesta granule	petroleum ether, ethyl acetate, ethanol	Petroleum ether: LC_{50} =7.81 mg/L Ethyl acetate: LC_{50} = 27.33 mg/L Ethanol: LC_{50} = 64.14 mg/L	Ginkgolic acids isolated from pretroleum extract caused snail mortalities to be 45% (C13:0), 65% (C15:1), 0% (C17:1); exposure time = 72 h; concentration of extract = 2 mg/L	[138]
	Leaf	Ethanol	Ethanol: 100% mortality caused after 72 h exposure at a concentration of 100 mg/L	Petroleum ether fractioned of the ethanolic extracts caused 100% mortality at a dose of 35 mg/L; exposure time: 72 h	[121]

Table 6. *Cont.*

Species Name	Plant Parts Used	Extraction Solvent	Activity (%Mortality/ LC or LD) Value	Active Compounds	References
Hemerocallis fulva	Root	Ethanol	76.25% mortality caused after 72 h at a concentration of 200 mg/L		[136]
Herba agrimoniae	Herb	Ethanol	80% mortality caused after 72 h exposure; dose = 100 mg/L	phorbol esters	[121]
Jatropha curcas	Seeds	Water extract	Around 50% snail mortality caused from 48 exposure at a concentration of 0.03% (*w*/*v*)		[139]
		Oil was produced from nuts by pressure and phorbol esters were enriched by extracting five time with an equal volume of methanol.			
Juglis cathayensis var. *formosana*	Fruit	Ethanol	60% mortality caused after 72 h exposure at a concentration of 100 mg/L		[136]
Macleaya cordata (Willd) R. Br	Leaf	Ethanol	1–7 alkaloid components were evaluated for molluscicidal activity. Highest activity (73.33% mortality) observed after 48 h exposure with 28 mg/L of Alkaloid component 2 LC_{50} and LC_{90} values of Alkaloid component 2 = 6.35 and 121.23 mg/L, respectively		[135]
		Total Alkaloid was extracted			
Nerium indicum Mill	Leaf	N-butanol, Water	Exposure time: 48 h N-butanol: LD_{50} =16.2 mg/L Water: LD_{50} =13.2 mg/L		[140]
Peucedanum praerutorum	Root	Ethanol	83.33% mortality caused after 72 h exposure; dose = 100 mg/L		[121]
Pterocarya Stenoptera DC	Leaf	N-butanol, Water	Exposure time = 48 h N-butanol: LD_{50} = 505.1 mg/L Water: LD_{50} = 359.5 mg/L		[140]
Pulsatilla chinensis (Bunge) Regel	Root	Water	LC_{50}: 0.48 mg/L; exposure time = 24 h	hederacochiside C, hederacolchiside A1	[132–134]

Table 6. *Cont.*

Species Name	Plant Parts Used	Extraction Solvent	Activity (%Mortality/ LC or LD) Value	Active Compounds	References
Rheum palmatum	Root tubers	Water	> 50% snail mortality was achieved after 48 h exposure with 0.2% (*v*/*v*)	Anthraquinones including rhein, chrysophanol-anthron, rheum-emodin and physcion	[139]
Rhumex dentatus	Root tubers	Water	> 50% snail mortality was achieved after 48 h exposure with 0.2% (*v*/*v*)	Anthraquinones including rhein, chrysophanol-anthron, rheum-emodin and physcion	[139]
Rhinacanthus nasutus	herb	Ethanol	81.25% mortality caused after 72 h exposure with 200 mg/L extract		[136]
Rumex japonicum	Roots	N-butanol and Water crude extracts	Exposure time = 48 h N-butanol: LD_{50} =398.1 mg/L Water: LD_{50} = 90.0 mg/L		[140]
Sapium sebiferum	fruit	Ethanol	55% mortality caused after 72 h exposure with 200 mg/L extract		[136]
Solanum xanthocarpum (Schrad and Wendl)	Fruit	Ethanol	100% snail mortality was achieved after 48 h exposure with 4.321 mg/L extract		[111]
			LC_{50} = 0.181 mg/L for 72h of exposure		[141]
Torreya grandis	Leaf	Ethanol	80% mortality caused after 72 h exposure; dose= 100 mg/L		[121]

6. Conclusions

Schistosomiasis is a worldwide disease and needs the integration of several measures to promote its control, which includes clinical treatment with antiparasitic agents, as well as the use of molluscicides to promote the biological control. For these purposes, the use of plant-based actives available in the affected countries is one of the viable, ecologically friendly and cheaper alternatives, since most of them are considered developing countries. Thus, this review demonstrates that several researches with natural resources present a broad diversity of species/substances that have action in different phases of the schistosomiasis cycle and can conduct further studies with active formulations and optimization of the best candidates to be used in the combat against this disease in each country.

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Salvia Spp. Essential Oils against the Arboviruses Vector *Aedes albopictus* (Diptera: Culicidae): Bioactivity, Composition, and Sensorial Profile—Stage 1

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Abstract: Mosquito-borne arboviruses diseases cause a substantial public health burden within their expanding range. To date, their control relies on synthetic insecticides and repellents aimed to control the competent mosquito vectors. However, their use is hampered by their high economic, environmental, and human health impacts. Natural products may represent a valid eco-friendly alternative to chemical pesticides to control mosquitoes, and mosquito-borne parasitic diseases. The aim of this work was to combine the chemical and sensorial profiles with the bioactivity data of *Salvia* spp. essential oils (EOs) to select the most suitable EO to be used as a repellent and insecticide against the invasive mosquito *Aedes albopictus* (Diptera: Culicidae), vector of pathogens and parasites, and to describe the EOs smell profile. To do this, the EOs of four *Salvia* species, namely *S. dolomitica*, *S. dorisiana*, *S. sclarea*, and *S. somalensis* were extracted, chemically analyzed and tested for their bioactivity as larvicides and repellents against *Ae. albopictus*. Then, the smell profiles of the EOs were described by a panel of assessors. The LC_{50} of the EOs ranged from 71.08 to 559.77 μL L^{-1} for *S. dorisiana* and *S. sclarea*, respectively. *S. sclarea* EO showed the highest repellence among the tested EOs against *Ae. albopictus* females (RD_{95} = 12.65 nL cm^{-2}), while the most long-lasting, at the dose of 20 nL cm^{-2}, was *S. dorisiana* (Complete Protection Time = 43.28 ± 3.43 min). *S. sclarea* EO showed the best smell profile, while *S. dolomitica* EO the worst one with a high number of off-flavors. Overall, all the EOs, with the exception of the *S. dolomitica* one, were indicated as suitable for "environmental protection", while *S. dorisiana* and *S. sclarea* were indicated as suitable also for "Body care".

Keywords: essential oil composition; insecticide; mosquitoes; mosquito-borne arboviruses diseases; repellent; sensory quality

1. Introduction

Mosquitoes (Diptera: Culicidae) are among the most serious threats for humans because of their ability to transmit viruses and parasites. In particular, the arboviruses dengue, yellow fever,

chikungunya, and Zika have recently expanded their geographical distributions and caused severe disease outbreaks in many urban populations [1–3]. Since the transmission of these viruses depends on the presence of the competent mosquito vectors *Aedes aegypti* and *Aedes albopictus* [3–5], measures such as repellent- or insecticide-treated nets, indoor spraying, or personal protection measures are needed to protect people from mosquito-borne infections [6]. To date, mosquitoes are mainly controlled by synthetic insecticides and repellents, but besides the quick development of resistance by insect pests, their use is often prohibitively expensive, unsustainable, and it poses relevant risks to humans and environmental health [7].

In this regard, essential oils (EOs) for their effectiveness, minimal toxicity to mammals, and low impact on the environment [8–10] have been recognized among the best alternative to synthetic chemicals. However, despite their insecticidal and repellent properties, EOs still do not have the expected broad use. In fact, besides the composition variability and the high volatility, their strong smell prevents their widespread application [11,12]. For these reasons, the acceptance of EOs to the human sensorial system is an important feature for their success as an ingredient in commercial products for topical use or for environmental protection.

The genus *Salvia* comprises many easily cultivable species well-known in traditional medicine, all around the world [13]. Besides the medicinal effects, *Salvia* spp. EOs have also been extensively reported for their antimicrobial and antifungal activity [14,15], as well as for their repellent and insecticidal properties [16–19].

The aim of this work was, therefore, to evaluate the efficacy as repellents and insecticides of the EOs extracted from four cultivated *Salvia* species, namely *S. dolomitica* Codd, *S. dorisiana* Standl., *S. sclarea* L., and *S. somalensis* Vatke, against the filariasis vector *Aedes albopictus* Skuse (Diptera: Culicidae). Besides, we described the EOs smell profile for their possible use as active ingredients in the formulation of products for the environment or for personal use.

2. Materials and Methods

2.1. Plant Material, EOs Extraction and GC-MS Analysis

The EOs were obtained from air-dried aerial parts of *Salvia dolomitica* Codd, *Salvia dorisiana* Standl., *Salvia sclarea* L., and *Salvia somalensis* Vatke (Lamiaceae) plants cultivated at the Centro di Ricerca Orticoltura e Florovivaismo, (CREA) (Sanremo, Italy) and collected during the Summer 2017 (Table S1). The EOs were obtained by extraction for 2 h in a Clevenger apparatus. After the hydro-distillation, the EOs were dehydrated using anhydrous sodium sulphate and stored at −4 °C until analysis. The EOs were chemically analyzed by gas chromatography-mass spectroscopy (GC–MS) by an Agilent 7890B gas chromatograph equipped with an Agilent HP-5MS capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and an Agilent 5977B single quadrupole mass detector (Agilent Technologies Inc., Santa Clara, CA, USA). Analytical conditions were as follows: carrier gas helium at 1 mL/min; injection of 1 μL (0.5% HPLC grade *n*-hexane solution); oven temperature programmed from 60 to 240 °C at 3 °C min^{-1}; split ratio 1:25, injector and transfer line temperatures 220 and 240 °C, respectively. The parameters that were acquired were as follows: full scan, scan range of 30–300 m/z; scan time of 1 sec. The identification of the constituents was based on a comparison of the retention times with those of the authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons. Computer matching was also used against commercial (NIST 14 and ADAMS) and laboratory-developed mass spectra library built up from pure substances and components of known oils and Mass Spectra (MS) literature data [20–24].

2.2. Aedes albopictus Rearing

Adults of *Aedes albopictus* Skuse (Diptera: Culicidae) were obtained from eggs collected in the open field on Masonite strips put in black pots filled with 1 L of tap water. The strips were daily collected, transferred in 500 mL beakers, and submerged in tap water under room conditions (26 ± 2 °C; 60%

relative humidity), photoperiod of 14:10 h (L:D), for eggs hatching. The emerged larvae were fed with cat food until pupation. The pupae (about 300 per cage) were put in cylindrical cages (Plexiglas, 35 d7 60 cm) with a front cotton access sleeve. The emerged adults were kept under room conditions and fed with sucrose solution (20%) [17,25].

2.3. EOs Larvicidal Activity

Ten newly fourth-instar larvae (0-24 h) were put in a 250 mL beaker with 0.1% Tween 80 water solutions of the EOs. The EOs were tested at 50, 100, 150, 200, 300, 400, and 600 μL L^{-1}. As a control, 10 larvae were put in 0.1% Tween 80 tap water solution. Four replicates for each treatment were performed. The mortality of the larvae was recorded after 24 h. During the tests, no food was given to the larvae [26]. Abbott's formula [27] was used to adjust the mortality percentage rates of the treatments on the basis of the controls' mortality.

2.4. Essential Oils Repellent Activity

The repellence of the EOs was evaluated by the human-bait technique [28] with some modifications. The experiments were performed during the summer in the above-described cages. The cages contained about of 300 8–12 day-old adults (sex ratio 1:1). The mosquitoes were starved for 12 h and were not blood-fed or exposed to any form of repellent. The tests were performed by ten volunteers not allergic to mosquito bites, and that had no contact with perfumed products on the day of the bioassay. All volunteers were informed about the experiment and provided their written consent. After rinsing the hands in distilled water, the volunteer's forearms were protected with thick fabric sleeves and the hands with latex gloves in which a dorsal square area 5 × 5 cm was cut open. The mosquito-exposed skin of one hand was treated with 100 μL of ethanol as a negative control. The other hand was treated with 100 μL of EO ethanolic solution at concentrations ranging from 0.02 to 200 nL cm^{-2}. After ethanol evaporation, the control hand was inserted inside the cage and exposed to mosquitoes for 3 min. Immediately after, the other hand was treated with the EO solution and, after ethanol evaporation, exposed to mosquitoes in the same cage. The number of probing mosquitoes was recorded by two observers. All the tests were performed between 8:00 and 10:00 am. The complete protection time (CPT) for the concentration of the EO of 0.2 μL cm^{-2} of skin was calculated by tests performed every 15 min until either two bites occurred in a single exposure period or one bite occurred in each of two consecutive exposure periods. The complete protection times were calculated on an average of six replicates. To verify the mosquitoes' readiness to bite, the control and the EOs treated hand were regularly interchanged during each test. We considered the test valid if at least 30 mosquitoes landed on the control hand and attempted to bite. If the number of probing was < 30, a new mosquito's cage was used [11]. The study was approved by the ethical committee of the University of Pisa (Comitato Bioetico dell'Università di Pisa).

2.5. Essential Oils Sensory Analysis

The sensory analysis of the EOs was performed by a panel of 10 assessors (four males and six females aged from 23 to 60 years) selected and trained for sensory analysis of foods (mainly wine, vegetal oils, and bakery products) and non-foods (mainly essential oils), according to the internal protocol of the Department of Agriculture, Food, and Environment (DAFE) of University of Pisa [29,30]. For this general training protocol, five training sessions specific for the assessment of *Sage* spp. were arranged until the assessors familiarized with the main descriptors useful for the characterization of aromatic plants. With this aim, during these training sessions, assessors were asked to identify and describe the smell profile of different solutions prepared by infusion (12 h, 25 °C of temperature, inert atmosphere) in hydroalcoholic solution (13% *v*/*v*) of the main aromatic plants, spices, different flowers, fruits, and fresh vegetables.

The smell assessment of the EOs tested was performed as a blinded test, in a quiet, well-ventilated room in the morning. Each assessor was provided with a filter paper (2 × 2 cm) soaked with 20 μL of

EO (1%). To avoid cross-contamination, the five samples were assessed separately in the same panel session (15 min waiting between two tests). The assessors were provided with a specific non-structured parametric descriptive scoring chart, and described the main odors of each sample on the basis of descriptors ranked on a scale of 0–10 in terms of "Smell intensity", "Smell persistency", and, "Overall pleasantness" as hedonic parameters [31]. The assessors also evaluated the possible use (Body care or Environmental protection) of the EOs, as well as the main emotions (Familiar, Relax, Exotic, Repulsion) elicited by them.

2.6. Statistical Analysis

Data of the smell profiles assessments of EOs were analyzed by one-way ANOVA with the EO as factor. Equality of variances was checked before the analyses by the Levene's test. The averages were separated by the Tukey's b post-hoc test. Larvae median and lethal concentration to 95% of tested organisms (LC_{50}, LC_{95}), and adult median and total repellent dose (RD_{50}, RD_{95}) were calculated by probit regression. Differences between LC_{50}, LC_{95}, RD_{50}, and RD_{95} values were evaluated by the relative median potency (rmp). The complete protection time (CPT) data were processed by the Kruskal–Wallis test with the time of protection as a factor. The means were separated by Dunn-Bonferroni pairwise comparisons. All the analyses were performed by the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Essential Oils Chemical Composition

In total, 108 compounds were identified in the four *Salvia* spp. EOs, with an identification percentage ranging from 96.9% to 99.8% (Table 1).

The results showed that almost all the EOs were characterized by the total monoterpenes (from 52.84 to 90.87% in *S. dorisiana* and *S. sclarea*, respectively) as the main class of constituents, even though differently distributed among hydrocarbons and oxygenated derivatives. *Salvia sclarea* exhibited the highest percentage of oxygenated monoterpenes (68.46%), while *S. dorisiana* and *S. somalensis* pointed out nearly a similar amount in oxygenated monoterpenes (Table 1). The highest percentage of non-terpene derivative (20.66%) was detected in the *S. dorisiana* EO. On the other hand, *S. dolomitica* evidenced the highest percentage of sesquiterpenoids (51.06% of sesquiterpene hydrocarbons and 12.33% of oxygenated sesquiterpenes). Regarding the main identified constituents, both bornyl acetate and camphor were the main constituents of *S. somalensis* EO (18.10% and 12.91%, respectively) (Table 1). This latter species also evidenced an interesting amount of α-pinene (6.77%), camphene (6.05%), δ-3-carene (5.19%), β-caryophyllene (3.62%), τ-cadinol (5.12%), and limonene (4.13%).

Salvia dolomitica was characterized by the highest amount of β-caryophyllene (14.81%) followed by eucalyptol (10.17%) and aromadendrene (7.96%). δ-cadinene, borneol, γ-cadinene and, viridiflorene showed also a good relative percentage (5.86 > 4.41 > 4.36 > 3.96%, respectively), and β-eudesmene and α-eudesmol were exclusive constituents of this EO.

Salvia dorisiana EO showed a different composition, in fact, it was rich in perillyl acetate (21.74%) and methyl perillate (19.16%), together with β-caryophyllene (9.99%) and myrtenyl acetate (4.03%). It is interesting to note that perillyl acetate, methyl perillate, and myrtenyl acetate were present only in *S. dorisiana* EO, Linalyl acetate (32.03%) and α-thujene (9.91%) characterized *S. sclarea* EO, followed by linalool (11.90%).

Table 1. Chemical composition of *Salvia dolomitica*, *S. dorisiana*, *S. sclarea*, and *S. somaliensis* essential oils.

N°	Compounds	Rt	Class	LRI [1]	LRI [2]	*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
						Relative Percentage (%)			
1	Tricyclene	5.28	MH	925	921	-	-	0.10	0.29
2	α-Thujene	5.36	MH	930	924	-	-	9.91	-
3	α-Pinene	5.55	MH	937	932	2.22	2.53	3.87	6.77
4	Camphene	5.94	MH	952	946	0.65	1.24	1.79	6.05
5	Sabinene	6.58	MH	978	968	0.21	-	0.10	-
6	β-Pinene	6.69	MH	979	974	1.23	0.56	2.86	0.75
7	Myrcene	7.05	MH	991	988	1.72	1.19	0.40	1.07
8	α-Phellandrene	7.49	MH	1005	1002	0.33	0.32	-	0.48
9	δ-3-carene	7.68	MH	1011	1008	3.28	2.16	-	5.19
10	1,4-Cineole	7.81	OM	1016	1012	-	-	0.49	-
11	α-Terpinene	7.88	MH	1017	1014	0.14	0.27	0.22	0.45
12	*o*-Cymene	8.00	MH	1022	1020	2.02	0.98	-	0.10
13	*p*-Cymene	8.14	MH	1025	1022	-	-	1.65	1.76
14	Limonene	8.28	MH	1030	1024	3.81	3.70	1.34	4.13
15	Eucalyptol	8.37	OM	1032	1026	10.17	5.83	4.96	-
16	*cis*-β-Ocimene	8.55	OM	1038	1032	1.58	0.87	-	-
17	*trans*-β-Ocimene	8.91	OM	1049	1044	0.16	0.23	0.10	-
18	γ-Terpinene	9.31	MH	1060	1054	0.26	0.49	0.17	0.53
19	*cis*-4-Thujanol	9.56	OM	1070	1074 [$]	0.11	-	-	-
20	*cis*-Linalool oxide (furanoid)	9.79	OM	1074	1067	-	-	0.10	-
21	2-methoxyethyl-Benzene	10.27	NT	1087	1080	0.15	-	-	-
22	Terpinolene	10.38	MH	1088	1086	-	0.29	-	0.84
23	Fenchone	10.40	OM	1096	1083	0.45	-	0.45	-
24	Linalool	10.78	OM	1099	1095	-	0.23	11.9	0.21
25	*iso*-Amyl 2-methyl butyrate	11.03	NT	1101	1100 [$]	-	0.26	-	-
26	β-Thujone	11.45	OM	1114	1112	-	-	2.40	-
27	*trans*-Sabinol	12.35	OM	1143	1137	0.39	-	-	-
28	Camphor	12.55	OM	1145	1141	0.27	-	8.10	12.91
29	Borneol	13.38	OM	1167	1165	4.41	3.61	1.08	3.35
30	4-Terpineol	13.86	OM	1177	1174	0.61	0.49	0.20	0.79
31	*p*-Cymen-8-ol	14.16	OM	1183	1179	-	-	-	0.17
32	α-Terpineol	14.14	OM	1189	1186	0.41	0.48	2.93	1.87
33	Myrtenol	14.64	OM	1195	1194	-	0.49	-	-

Table 1. *Cont.*

N°	Compounds	Rt	Class	LRI [1]	LRI [2]	*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
						Relative Percentage (%)			
34	γ-Terpineol	14.68	OM	1197	1199	-	-	0.18	-
35	Nerol	15.97	OM	1228	1227	-	-	0.22	-
36	Linalyl acetate	17.18	OM	1257	1254	-	-	32.03	-
37	Bornyl acetate	18.41	OM	1285	1284	-	-	0.84	18.1
38	*p*-Mentha-1,8-dien-7-ol	18.89	OM	1296	1297 [$]	-	1.11	-	-
39	Carvacrol	19.07	OM	1299	1298	-	-	0.47	-
40	Myrtenyl acetate	20.06	OM	1327	1324	-	4.03	-	-
41	α-Cubebene	21.06	SH	1351	1345	0.76	-	-	0.22
42	Eugenol	21.36	OM	1357	1356	-	-	0.23	-
43	Neryl acetate	21.71	OM	1364	1359	-	-	0.74	-
44	Ylangene	21.96	SH	1372	1373	-	-	-	0.52
45	Isoledene	22.03	SH	1375	1374	0.5	-	-	-
46	α-Copaene	22.15	SH	1376	1374	2.76	0.82	0.27	2.59
47	Geranyl acetate	22.51	OM	1382	1379	-	-	1.04	-
48	β-Cubebene	22.74	SH	1389	1387	0.10	-	-	-
49	*cis*-Jasmone	23.02	NT	1393	1392	-	-	-	0.37
50	Methyl perillate	23.23	NT	1394	1392	-	19.16	-	-
51	β-Panasinsene	23.33	SH	1395	1381	0.17	-	-	-
52	β-Maaliene	23.45	SH	1405	1411 [$]	-	-	-	0.57
53	α-Gurjunene	23.53	SH	1410	1409	1.17	-	-	-
54	β-Caryophyllene	23.92	SH	1419	1417	14.81	9.99	3.47	3.62
55	β-Gurjunene	24.27	SH	1432	1431	1.46	-	-	0.15
56	1,1,3a-Trimethyl-7-methylenedecahydro-1H-cyclopropa[a]naphthalene	24.56	SH	1434	1435	-	0.18	-	-
57	*trans*-α-Bergamotene	24.60	SH	1435	1432	-	-	0.12	-
58	*p*-Mentha-1,8-dien-7-yl acetate	24.69	OM	1436	1436 [$]	-	21.74	-	-
59	Aromadendrene	24.84	SH	1440	1439	7.96	-	-	1.00
60	α-Maaliene	24.89	SH	1443	1442 [$]	0.96	-	-	0.12
61	Selina-5,11-diene	25..12	SH	1447	1447 [$]	0.95	0.15	-	-
62	α-Humulene	25.29	SH	1454	1452	1.57	0.76	2.22	0.27
63	Cadina-3,5-diene	25.32	SH	1458	1454 [$]	-	-	-	0.45
64	Alloaromadendrene	25.58	SH	1461	1458	0.87	0.19	-	0.33
65	γ-Muurolene	26.24	SH	1477	1478	0.72	0.17	-	1.10

Table 1. *Cont.*

N°	Compounds	Rt	Class	LRI [1]	LRI [2]	*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
						Relative Percentage (%)			
66	α-Amorphene	26.32	SH	1482	1483	-	-	-	0.17
67	Germacrene D	26.40	SH	1485	1484	-	-	0.10	-
68	β-Eudesmene	26.51	SH	1486	1485 [$]	1.02	-	-	-
69	Phenethyl isovalerate	26.79	NT	1490	1491 [$]	-	0.18	-	-
70	δ-Selinene	26.83	SH	1493	1492	0.27	-	-	-
71	*epi*-Bicyclosesquiphellandrene	26.85	SH	1494	1490 [$]	-	-	-	0.42
72	Viridiflorene	27.00	SH	1497	1496	3.96	0.75	0.10	-
73	Eremophilene	27.07	SH	1498	1498 [$]	-	-	-	0.63
74	α-Muurolene	27.11	SH	1499	1500	0.53	-	-	0.99
75	β-Bisabolene	27.52	SH	1509	1505	-	-	0.10	-
76	γ-Cadinene	27.81	SH	1513	1513	4.36	1.04	-	2.67
77	δ-Cadinene	28.09	SH	1524	1522	5.86	2.18	0.11	5.66
78	Cubenene	28.48	SH	1532	1522 [$]	0.12	-	-	0.48
79	α-Cadinene	28.61	SH	1538	1537	0.18	-	-	0.17
80	α-Calacorene	28.81	SH	1542	1544	-	0.13	-	0.55
81	Myrtenyl 2-methyl butyrate	29.55	NT	1560	1559 [$]	-	0.20	-	-
82	(*E*)-Nerolidol	29.64	OS	1563	1561	-	0.27	-	1.49
83	Spathulenol	30.12	OS	1576	1577	0.50	0.13	-	-
84	Globulol	30.36	OS	1580	1590	3.36	-	-	0.57
85	Caryophyllene oxide	30.39	OS	1583	1582	-	1.62	0.94	-
86	Viridiflorol	30.71	OS	1591	1592	0.25	-	0.67	0.37
87	Ledol	30.99	OS	1599	1602	0.37	-	-	-
88	Rosifoliol	31.12	OS	1600	1600	1.55	-	-	-
89	Humulene epoxide II	31.31	OS	1606	1608	0.17	0.10	0.26	-
90	Di-*epi*-1,10-cubenol	31.54	OS	1614	1618	0.18	-	-	0.18
91	Junenol	30.77	OS	1617	1618	-	0.53	-	1.36
92	(*E*)-Farnesene epoxide	30.89	OS	1624	1624 [$]	-	0.35	-	-
93	Epicubenol	31.00	OS	1627	1627	0.63	-	-	-
94	γ-Eudesmol	31.16	OS	1632	1630	0.33	-	-	-
95	τ-Cadinol	32.52	OS	1640	1638	2.18	2.42	-	5.12
96	δ-Cadinol	32.66	OS	1645	1646 [$]	0.19	-	-	-

Table 1. *Cont.*

N°	Compounds	Rt	Class	LRI [1]	LRI [2]	*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
						Relative Percentage (%)			
97	octahydro-2,2,4,7a-tetramethyl-1, 3a-ethano(1H)inden-4-ol	32.78	OS	1648	1648 [$]	-	0.33	-	-
98	α-Cadinol	32.93	OS	1653	1652	-	0.17	-	0.28
99	α-Eudesmol	32.99	OS	1655	1652	2.48	-	-	-
100	Aromadendrene oxide-(2)	33.97	OS	1678	1678 [$]	-	0.16	-	-
101	α-Bisabolol	34.12	OS	1684	1683	-	0.26	-	-
102	Shyobunol	34.56	OS	1701	1686 [§]	0.14	0.25	-	-
103	Farnesyl acetone	42.13	AC	1919	1913	-	0.11	-	-
104	Cembrene	43.63	DH	1939	1937	-	0.23	-	-
105	(*E*)-1-(6,10-Dimethylundec-5-en-2-yl)-4-methylbenzene	44.02	NT	1951	1950 [$]	-	0.86	-	-
106	Gerany-*p*-cymene	44.98	DH	1980	1980 [$]	-	0.11	-	-
107	*epi*-13-Manool	48.77	OD	2056	2059	-	-	0.10	-
108	Sclareol	51.14	OD	2227	2222	-	-	0.43	-
	Class of compounds					*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
	Monoterpene Hydrocarbons (MH)					15.87	13.73	22.41	28.41
	Oxygenated Monoterpenes (OM)					18.56	39.11	68.46	37.40
	Total monoterpenes					34.43	52.84	90.87	65.81
	Sesquiterpene Hydrocarbons (SH)					51.06	16.36	6.49	22.68
	Oxygenated Sesquiterpenes (OS)					12.33	6.59	1.87	9.37
	Total sesquiterpenes					63.39	22.95	8.36	32.05
	Diterpene Hydrocarbons (DH)					-	0.34	-	-
	Oxygenated Diterpenes (OD)					-	-	0.53	-
	Apocarotenoids (AC)					-	0.11	-	-
	Non-terpene Derivatives (NT)					0.15	20.66	-	0.37
	Total Identified					97.97	96.90	99.76	98.23

LRI [1]: Linear retention indices on DB-5 column. LRI[2]: Linear retention indices reported by Adams 1995; [$]: Linear retention indices in NIST 2014 (https://webbook.nist.gov/chemistry/name-ser/); [§]: Linear retention indices in pherobase (www.pherobase.com/database/kovats).

3.2. Essential Oils Larvicidal Activity

The *Salvia* EOs showed a wide range of toxicity against the Asian tiger mosquito larvae, depending on the species. Overall, the toxicity of *S. dorisiana* EO (LC_{50} = 71.08 μL L^{-1}) was in line with the toxicity previously reported for other aromatic plants' EOs (LC_{50} ranging from 35 to 194 μL L^{-1}) (reviewed by Pavela, 2015), while a much lower toxicity against *Ae. albopictus* larvae was recorded for *S. dolomitica*, *S. dorisiana*, and *S. somalensis*. (LC_{50} ranging from 315.52 to 559.77 μL L^{-1}) (Table 2).

Table 2. Toxicity of *Salvia dorisiana*, *S. dolomitica*, *S. sclarea*, and *S. somalensis* essential oils (EOs) against larvae of *Aedes albopictus*.

EO	LC_{50} [a]	LC_{95} [b]	$\chi 2$ (df)	*P*
S. dolomitica	315.52 (293.24–338.14)	503.04 (454.85–582.09)	3.50 (8)	0.899
S. dorisiana	71.08 (65.91–76.14)	125.52 (112.70–146.54)	4.83 (8)	0.775
S. sclarea	559.77 (470.17–718.35)	2159.94 (1457.00–3974.90)	5.68 (8)	0.683
S. somalensis	388.51 (356.59–430.74)	686.63 (581.29–912.44)	0.99 (8)	0.998

[a], Concentration of the EO that kills 50% of the exposed larvae; [b], concentration of the EO that kills 95% of the exposed larvae. Data are expressed as μL L^{-1}; in bracket, confidence interval; df, degrees of freedom. *P*, significance level of Pearson goodness of fit test.

The comparison of the relative toxicity of *Salvia* spp. EOs against *Ae. albopictus* by rmp analysis of probits showed that the *S. dorisiana* EO was significantly more toxic to the mosquitoes' larvae than the other *Salvia* EOs. In addition, *S. dolomitica* was significantly more toxic than *S. sclarea* EO (Table 3).

Table 3. Relative toxicity of *Salvia dorisiana*, *S. dolomitica*, *S. sclarea*., and *S. somalensis* essential oils (EOs) against *Aedes albopictus* larvae.

EO (X) / EO (Y)	*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
S. dolomitica	-	**5.48(3.00–13.24)**	**0.69 (0.45–1.01)**	0.73 (0.46–1.12)
S. dorisiana	**0.18 (0.08–0.33)**	-	**0.13 (0.05–0.25)**	**0.13 (0.05–0.27)**
S. sclarea	**1.46 (1.01–2.23)**	**7.98(4.07–21.81)**	-	1.07 (0.71–1.62)
S. somalensis	1.37 (0.90–2.18)	**7.49(3.77–20.47)**	0.94 (0.62–1.42)	-

Relative median potency analyses (rmp) values of probits (EO in column vs. EO in row): Values < 1 indicate higher repellence. Values > 1 indicate lower repellence. Bold indicates significant values (95% CI ≠ 1).

3.3. Essential Oils Repellent Activity

All the *Salvia* spp. EOs showed a repellent activity against the *Ae. albopictus* adults. RD_{50} values ranged from 0.56 to 5.03 nL cm^{-2} for *S. dorisiana* and *S. somalensis*, respectively while RD_{95} values ranged from 12.65 to 8308.54 nL cm^{-2} for *S. sclarea* and *S. somalensis*, respectively (Table 4).

Table 4. Repellence of *Salvia dorisiana*, *S. dolomitica*, *S. sclarea*, and *S. somalensis* essential oils (EOs) against *Aedes albopictus* females.

EO	RD_{50} [a]	RD_{95} [b]	$\chi 2$ (df)	*P*
S. dolomitica	0.98 (0.62–1.54)	38.68 (18.50–86.26)	66.35 (7)	<0.001
S. dorisiana	0.56 (0.19–1.11)	39.88 (7.84–46986.17)	12.59 (3)	0.006
S. sclarea	1.13 (0.74–1.74)	12.65 (6.12–52.92)	28.36 (4)	<0.001
S. somalensis	5.03 (3.69–7.01)	8308.54 (3387–25371.72)	14.91 (8)	0.061

[a], dose of EO that reduces the number of landings to 50%; [b], dose of EO that reduces the number of landings to 95%. Data are expressed as nL cm^{-2} of skin. In bracket, confidence limits. *P*, significance level of Pearson goodness of fit test. Since $P < 0.150$, a heterogeneity factor is used in the calculation of confidence limits.

The comparison of the relative toxicity of *Salvia* spp. EOs by rmp analyses did not show significant differences among the EOs, with the exception of *S. somalensis* EO that was significantly less repellent than the others (Table 5).

Table 5. Relative repellence of *Salvia dorisiana*, *S. dolomitica*, *S. sclarea*, and *S. somalensis* essential oils (EOs) against *Aedes albopictus* females.

EO (X) / EO (Y)	*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
S. dolomitica	-	1.78 (0.56–6.02)	0.87 (0.29–2.63)	0.25(0.09–0.61)
S. dorisiana	0.56 (0.17–1.80)	-	0.49 (0.13–1.80)	0.14(0.04–0.45)
S. sclarea	1.14 (0.38–3.50)	2.04 (0.55–7.97)	-	0.29(0.09–0.83)
S. somalensis	4.00 (1.63–10.68)	7.07 (2.22–26.29)	3.46 (1.21–10.87)	-

Relative median potency analyses (rmp) values of probits (EO in column vs. EO in row): Values < 1 indicate higher repellence, values > 1 indicate lower repellence. Bold indicates significant values (95% CI ≠ 1).

The results of the complete protection time (CPT) assay indicated that the *Salvia* EOs applied to human skin at the dose of 20 nL cm^{-2} protect from the mosquito's bites for a time ranging from 4.60 ± 2.7 to 43.28 ± 3.43 min (for *S. somalensis* and *S. dorisiana*, respectively) (Table 6). The Kruskas–Wallis test indicated significant differences in the duration of the protection by *Salvia* EOs ($\chi 2 = 14.432$; df = 4; $P = 0.006$). The Dunn–Bonferroni pairwise comparisons of the CPT values indicated that the protective effect of *S. dorisiana* was significantly longer-lasting than the one of the *S. somalensis* EO (Table 6).

Table 6. Complete protection time (CTP) of *Salvia dorisiana*, *S. dolomitica*, *S. sclarea*, and *S. somalensis* essential oils (EOs) against *Aedes albopictus* females.

EO.	CPT [a]
S. dolomitica	21.45 ± 7.12 [ab]
S. dorisiana	43.28 ± 3.43 [a]
S. sclarea	13.60 ± 2.31 [ab]
S. somalensis	4.60 ± 2.70 [b]

[a], Complete protection time values (min) of *Salvia* EOs applied on human skin at the dose of 20 nL cm^{-2}; different letters indicate significant differences among the same dose of each EO (Kruskas–Wallis, Dunn–Bonferroni pairwise comparisons, $P \leq 0.05$).

3.4. Essential Oils Sensory Analysis

The panel tests indicated *S. dorisiana* as the EO with the highest "Smell intensity" (Figure 1), closely followed by *S. dolomitica* and *S. somalensis* as the EO with the lowest intensity. Such differences, however, were not statistically significant ($F_{(4.25)} = 2.12$. $p = 0.109$). On the contrary, the assessors attributed to the different EOs a significantly different "Smell persistency" as well as "Overall pleasantness" ($F_{(4.25)} = 5.80$, $p = 0.002$; $F_{(4.25)} = 10.83$, $p < 0.001$, respectively).

As for smell persistency, the highest value was attributed to *S. dorisiana*, followed by *S. dolomitica* and *S. sclarea* EO, with the lowest values attributed to the *S. somalensis* EO (Figure 1).

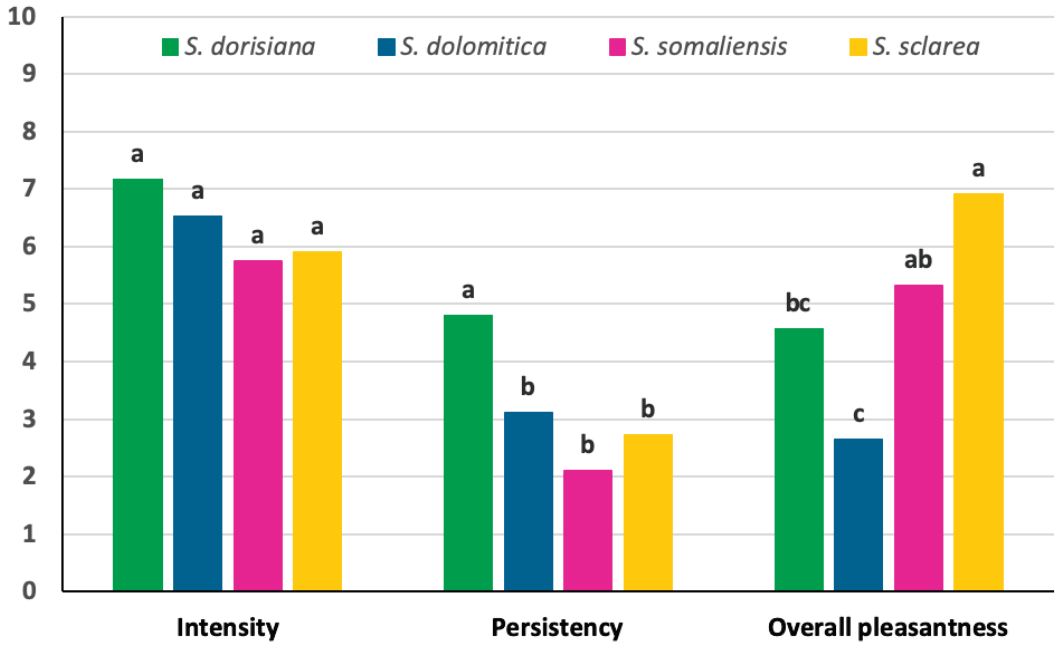

Figure 1. Smell characterization of the *Salvia dorisiana*, *S. dolomitica*, *S. sclarea*, and *S. somalensis* essential oils.

The best smell profile (highest value of overall pleasantness) was attributed to the *S. sclarea* EO, followed by the *S. somalensis* and the *S. dorisiana* EOs. The worst smell profile was attributed to the *S. dolomitica* EO that was characterized by a high number of off-flavors (Table 7).

Table 7. Main odors that characterized the smell of *Salvia dolomitica*, *S. dorisiana*, *S. sclarea*, and *S. somalensis* essential oils.

Species / Odour Class	*S. dolomitica*	*S. dorisiana*	*S. sclarea*	*S. somalensis*
Vegetative odours	Herbaceous	Herbaceous	Citronella	Herbaceous
	-	Mint	Fresh mint	Menthol
	-	-	Citrus	Chamomille
	-	-	Lime	-
Spicy	-	Sandalwood	Green spicy	Green spicy
	-	Licorice	Thyme	Thyme
	-	-	Sage	Green tea
Other	-	Resin	-	-
Off-flavors	Mould	-	-	-
	Wet rag	-	-	-
	Old soap	-	-	-
	Petrol	-	-	-

Furthermore, *S. sclarea* and *S. somalensis* EOs showed the highest complexity in flavor descriptors (Table 7) referring to both family of vegetative and Spicy odors.

All the assessors associated the *S. dolomitica* EO to negative emotion "Repulsion". On the contrary, the other *Salvia* spp. EOs were associated with positive sensations "Familiar" and "Relax" together with an "Exotic" feeling.

As for the possible use of the EOs, the assessors indicated that all the *Salvia* spp. EOs, with the exception of *S. dolomitica*, were suitable for "Environmental protection", while only the *S. dorisiana* and *S. sclarea* were suitable also for "Body care" (Figure 2).

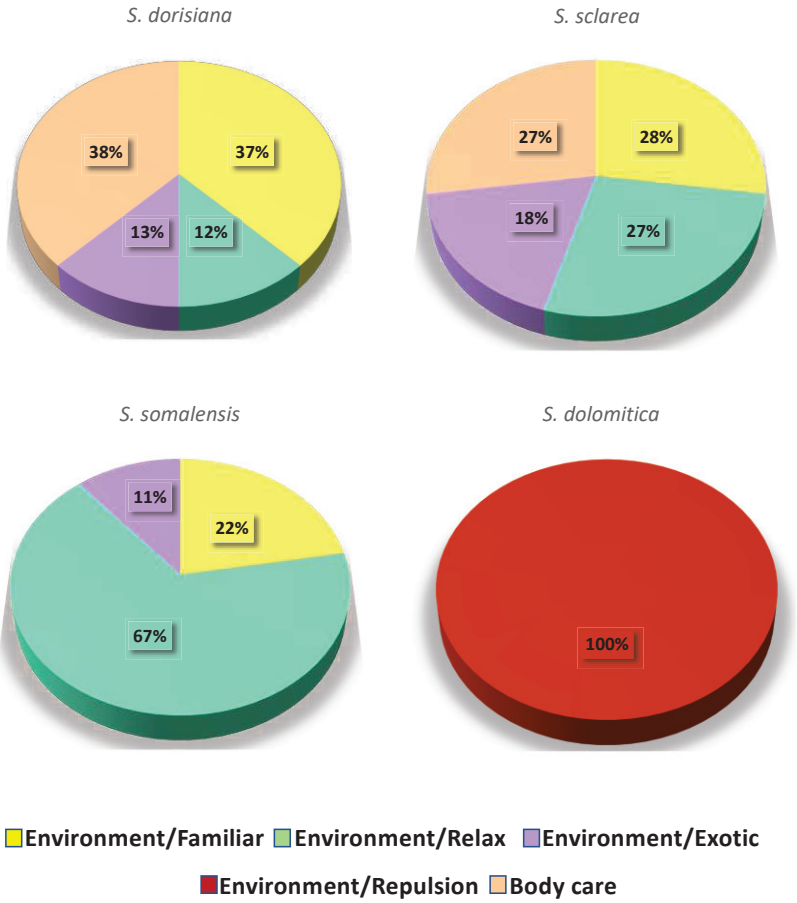

Figure 2. Main possible uses and emotions associated with the *Salvia dorisiana*, *S. dolomitica*, *S. sclarea*, and *S. somalensis* essential oils.

4. Discussion

The use of EOs as insecticides and insect repellents is raising increasing interest for their low impact on environmental and human health, and for their perception by consumers as safe, natural products [32]. As for their use as repellents, however, in order for them to be successfully utilized as an ingredient in commercial products for topical or for environmental protection use, the EOs need to have a good level of acceptance to the human sensorial system. In this work, we observed that the *Salvia* EOs tested showed a wide range of toxicity against the Asian tiger mosquito larvae, depending on the *Salvia* species and were able to show a repellent activity against the *Ae. albopictus* adults, depending on the concentration, while not all of them were indicated by the panelists as suitable to be utilized in the formulation of products for environmental protection or personal protection.

In order to get insights about the molecular components of the tested EOs that may act as chemical cues for mosquitoes and the human sensorial system, their chemical composition was characterized by GC-EIMS. The EO profiles of the studied species were completely in agreement with what was previously reported on *S. somalensis* [33] and *S. dorisiana* [17]. The *S. sclarea* EO evidenced results in partial agreement with those observed by Aćimović and co-workers [34] on clary sage EO from Tajikistan. The *S. dolomitica* EO was a subject of discord, in fact, Kamatou et al. [35] and Bassolino et al. [36] found a composition similar to what we found in the current study, while in a more recent study by Ebani [33], eucalyptol was the main compound. Caser underlined a completely different profile where limonene (19.8%), δ-3-Carene (9.1%), and germacrene D (8.6%) dominated together with (E)-β-ocimene (7.39%) and β-caryophyllene (7.9%) [37].

Overall, the toxicity of *S. dorisiana* EO against the *Ae. albopictus* larvae recorded in this work (LC_{50} = 71.08 μL L^{-1}) agrees with previous studies performed with other aromatic plants EOs (LC_{50}

ranging from 35 to 194 μL L^{-1}) (reviewed by Pavela [38]) and was shown to be significantly higher than the other *Salvia* spp. EOs. In line with our findings, a strong variability in the larval toxicity of *Salvia* spp. EOs was observed also by Ali [16] against the mosquitoes *Anopheles quadrimaculatus* and *Aedes aegypti* (Diptera: Culicidae). These results indicate a strong variability in the toxicity of the EOs among species of the genus *Salvia* that is consistent with the high variability in the chemical composition of the EOs.

Besides the high variability in the chemical composition of the EOs, a limitation in the use of the EOs as mosquito repellent is represented by their high volatility. This is why the determination of the protection time is an important parameter for the screening of EOs for possible practical use. In this experiment, *S. dorisiana* EO showed a very good persistency (CPT = 43 min at the dose of 20 nL cm^{-2} of skin) of the repellent effect that was longer than the one reported for the synthetic repellent *N*,*N*-Diethyl-*meta*-toluamide (DEET) (CPT = 10 min at the dose of 40 nL cm^{-2} of skin) [11]. Overall, *S. dorisiana* showed the highest toxicity against *Ae. albopictus* larvae coupled with the strongest protection effect against the *Ae. albopictus* bites that were about 60% longer than that of DEET. On the contrary, *S. somalensis*, was the least toxic and persistent EO among the *Salvia* species tested.

These results are in line with previous experiments by Conti et al. [17] who found that *S. dorisiana* and *S. sclarea* EOs were able to protect the human skin from *Ae. albopictus* bites up to 31 and 21 min (at 40 nL cm^{-2}), respectively. The repellent effect showed by *Salvia* spp. EOs in this work is consistent with one of EOs extracted from aromatic plants belonging to other families. For example, the EOs extracted from *Curcuma longa* L. (Zingiberaceae), *Pogostemon heyneanus* Benth. (Lamiaceae), and *Zanthoxylum limonella* Alston (Rutaceae) were found to repel the Asian tiger mosquito up to 23 min at a dose of 5% (about 10 times more concentrated than the solutions tested in the present study) [39]. Moreover, Nasir [40] found that the *Zingiber officinale* Rosc. (Zingiberaceae) *Mentha piperita* L., and *Ocimum basilicum* L. (Lamiaceae) showed 34–98 min of protection (EOs, 10%).

The sensorial analyses of the *Salvia* spp. EOs indicated that, overall, the best smell profile (highest value of overall pleasantness) was the one of the *S. sclarea* EO, followed by the *S. somalensis* and *S. dorisiana* EOs. The preference showed by the assessors to the *S. sclarea* EO should be probably due to the green spicy, fresh mint, menthol-like and citrus-like fragrances detected in all the samples. Those fragrances may be associated with the presence of the alcoholic volatile compounds' linalool and *p*-cymen-8-ol (Table 1).

On the contrary, the worst smell profile attributed to *S. dolomitica* EO is probably due to the off-flavors detected by panelists in EO that can be well explained by the presence in volatile fraction of borneol (smell character: camphoraceous odor [41]) in association with β-caryophyllene (smell character: dry-woody-spicy, clove-like odor [41]) and β-pinene (smell character: dry-woody, resinous odor [41]).

5. Conclusions

The control of mosquitoes is paramount in the efforts of stopping the spread of mosquito-borne diseases. According to our results, *Salvia* species may represent a valid source of repellents and insecticides alternative to the synthetic ones for the control of mosquitoes. In fact, unlike synthetic chemicals, the low-cost and the increasing demand for effective and safe natural products may make EO-based products well accepted by consumers, provided that they are compatible with their sensory system. The multidisciplinary approach of this study showed that the selection of EOs as ingredients for effective and good-smelling anti-mosquito formulations is feasible by combining the data of the biological activity with their chemical and smell profiles.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-7737/9/8/206/s1, Table S1: Main botanical characteristics, propagation and cultivation methods, plants main uses, and essential oils (EOs) main uses of *Salvia dorisiana*, *S. dolomitica*, *S. sclarea,* and *S. somaliensis*.

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Ethics Approval and Consent to Participate: All volunteers were informed on the experiment and provided written consent. The study was approved by the ethical committee of the University of Pisa (Comitato Bioetico dell'Università di Pisa).

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Cytotoxic, Antioxidant, and Enzyme Inhibitory Properties of the Traditional Medicinal Plant *Matthiola incana* (L.) R. Br.

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Abstract: *Matthiola incana* (L.) R. Br. (Brassicaceae) is widely cultivated for ornamental purposes and utilized as a medicinal plant. In the present work, the hydroalcoholic extract from the aerial parts of this species has been evaluated in different bioassays in order to detect potential pharmacological applications. The cytotoxic capacity against the human colorectal adenocarcinoma (CaCo-2) and breast cancer (MCF-7) cell lines was tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The extract was investigated as a neuroprotective inhibitor of central nervous system (CNS) enzymes such as monoamine oxidase A, tyrosinase, acetylcholinesterase, and as a natural enzyme inhibitor of α-glucosidase and lipase involved in some metabolic disorders such as obesity or type 2 diabetes. The antioxidant ability was also evaluated in an enzymatic system (xanthine/xanthine oxidase assay). Results showed that the *M. incana* extract displayed moderate to low cytotoxicity vs. CaCo-2 cells. The extract acted as a superoxide radical scavenger and enzymatic inhibitor of monoamine oxidase A, tyrosinase, α-glucosidase, and lipase. The best results were found in the α-glucosidase assay, as *M. incana* hydroalcoholic extract was able to inhibit the enzyme α-glucosidase up to 100% without significant differences, compared to the antidiabetic drug acarbose. *Matthiola incana* has been demonstrated to exert different biological properties. These are important in order to consider this species as a source of bioactive compounds.

Keywords: anti-glucosidase; antioxidant; Brassicaceae; medicinal plants; enzyme inhibitor; polyphenols; stock flower

1. Introduction

Matthiola incana (L.) R. Br. (Brassicaceae), usually called "stock flower", is widely cultivated for ornamental purposes in many regions around the world [1,2].

This species is utilized in the traditional medicine of several countries (Iran, India, Ecuador, Bolivia, Italy) for the treatment of various ailments, including inflammations and cancer, particularly breast and testicular cancer [3–11].

Matthiola incana is also utilized as an edible plant; the flowers are eaten as a vegetable or used as a garnish, especially with sweet desserts [7,12], and the freshly boiled pods are consumed in Italy (Puglia). In China, the edible flowers are sold in health food stores and consumed in the form of tea [13,14].

Considering all the above-mentioned uses of *M. incana*, our research team recently published a study reporting the phytochemical composition and the biological properties of a hydroalcoholic extract (80% methanol) obtained from the aerial parts (leaves and flower buds) of *M. incana* growing wild in Sicily, Italy. In our previous work, the phenolic compounds and volatile constituents were characterized for the first time. The extract was found to possess antioxidant activity in both in vitro and in vivo assays. In particular, it showed good chelating properties and a protective effect on *Escherichia coli* growth and survival from the oxidative stress induced by hydrogen peroxide. Moreover, the extract proved non-toxic against brine shrimp larvae (*Artemia salina* Leach), indicating its potential safety [15].

As part of the ongoing research, the present work was designed to assess further biological activities of the same extract using a wide variety of in vitro bioassays.

Starting from its traditional use in the prevention and treatment of different types of cancers, including breast cancer, the cytotoxicity of the extract against two different human cancer cell lines, breast (MCF-7) and colorectal adenocarcinoma (CaCo-2), was tested through the MTT (3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide) assay in order to provide a scientific basis for the empiric use of *M. incana*.

In recent years, ever-growing evidence indicates that oxidative stress plays a key role in the pathogenesis of a number of diseases associated with neurodegeneration and of many metabolic disorders [16,17]. Further, increasing evidence suggests a link between the incidence and progression of some neurodegenerative disorders and metabolic dysfunction [18]. Antioxidants can provide the desired antioxidant status, therefore contributing to the prevention of these pathologies; for this reason, oxidative stress is a therapeutic target. Indeed, several research studies have addressed the ability of natural antioxidants to delay or prevent neurodegenerative and metabolic disorders.

Taking into account the antioxidant activity of *M. incana* extract previously demonstrated, the second aim of this work is to assay the antioxidant capacity of the extract in an enzymatic system and to investigate its effect as a neuroprotective inhibitor of central nervous system (CNS) enzymes and as an enzyme inhibitor of α-glucosidase and lipase.

The ability to modulate key physiological enzymes has been investigated using monoamine oxidase A (MAO-A), tyrosinase (TYR), acetylcholinesterase (AChE), whose inhibition may lead to a neuroprotective effect [19–21], and lipase and α-glucosidase (α-GLU), to establish the extract's anti-obesity and antidiabetic potential [22].

2. Materials and Methods

2.1. Reagents and Chemicals

Xanthine, nitroblue tetrazolium (NBT), xanthine oxidase, gallic acid, galantamine, acetylthiocholine iodide (ATCI), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), tris, acetylcholinesterase from electric eel (AChE), vanillic acid, 4-aminoantipyrine, horseradish peroxidase, tyramine, monoamine oxidase A (MAO-A), levodopa (L-DOPA), tyrosinase (TYR), orlistat, lipase (type II) from porcine pancreas, p-nitrophenyl butyrate (pNPB), α-glucosidase (α-GLU) from *Saccharomyces cerevisiae*, and p-nitrophenyl glucopyranoside (pNPG) were acquired through Sigma-Aldrich (Madrid, Spain); clorgyline, kojic acid, and acarbose were from Cymit Quimica (Barcelona, Spain); Na_2CO_3, HCl, NaCl, $MgCl_2$, $CaCl_2$, MeOH, and potassium phosphate were from Panreac (Barcelona, Spain), and fetal bovine serum was from Gibco (Barcelona, Spain). All standards used for comparative purposes in the bioassays and experiments were acquired with a purity of at least 99%. All the chemicals and reagents not mentioned above were purchased from Sigma-Aldrich (Milano, Italy).

2.2. Plant Material and Extraction

The aerial parts (leaves and flower buds) of *Matthiola incana* (L.) R. Br. were collected in March, just before flowering, around Capo d'Orlando (Messina, Sicily, Italy). The taxonomic identification was confirmed by Prof. S. Ragusa, Department of Health Sciences, University "Magna Graecia" of Catanzaro. Voucher specimens were deposited in the Herbarium of the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, under accession number 406/17. After harvesting, the aerial parts were frozen, then the freeze-dried plant material (25.76 g) was subjected to preventive maceration with 80% MeOH (250 mL) for 150 min. The extraction was carried out with 80% MeOH (250 mL) in an ultrasonic bath at 50 °C for 15 min (three times). The filtrates were combined and evaporated to dryness by rotavapor; the yield of the extract, referred to 100 g of lyophilized plant material, was 33.33%.

2.3. Cytotoxic Activity

2.3.1. Cell Culture and Treatments

Human colorectal adenocarcinoma cells (CaCo-2), obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured as previously described [23]. The cells were plated at a constant density of 3×10^5/mL in order to obtain identical experimental conditions in the different tests and to achieve high accuracy of measurement. MCF-7 breast cancer cells (ATCC cell bank, Rockville, MD, USA) were cultured in RPMI medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 at 37 °C and seeded at a constant density of 3×10^5/mL. Both cell lines were seeded in 96-well plates for MTT assay and in 6-well plates for lactate dehydrogenase (LDH) release. At sub-confluent conditions, CaCo-2 and MCF-7 cells were treated with different concentrations of the *M. incana* extract for 48 and 72 h. The extract was dissolved in medium to obtain final concentrations ranging from 0.0625 to 4 mg/mL. After the treatments, cells were scraped, washed with PBS, and subsequently utilized for analysis.

2.3.2. MTT Bioassay

The MTT assay was performed according to Malfa and collaborators [24] in order to assess cell viability. After 24 h, cells were treated with different concentrations of extract (0.0625–4 mg/mL) for 48 and 72 h. The optical density was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories, Helsinki, Finland) at λ = 570 nm. The results were expressed as a percentage of cell viability with respect to control (untreated cells).

2.3.3. LDH Release

Necrotic cell death was measured by LDH release as a consequence of cell membrane disruption. Enzyme activity was measured, in cell culture medium and in the cellular lysates, spectrophotometrically at λ = 340 nm through the reduction of oxidized nicotinamide adenine dinucleotide (NAD) [23]. LDH release was calculated as a percentage of the total amount, as a sum of the enzymatic activity in the cellular lysate and in the culture medium. The results were expressed as a percentage of LDH released.

2.4. Antioxidant Activity: Superoxide Radical Scavenging Activity

The xanthine/xanthine oxidase assay was used to assess the ability of *M. incana* extract to eliminate superoxide radicals generated by the reaction [25]. The assay was performed in 96-well microplates, and the mixture contained 90 μM of xanthine, 16 mM of Na_2CO_3, 22.8 μM of NBT (solved in phosphate buffer, pH 7), and sample or reference inhibitor (gallic acid) at different concentrations. Then, xanthine oxidase (168 U/L) was added to start the reaction. The mixture was incubated for 2 min at 37 °C. Controls were performed in order to obtain 100% activity, containing buffer instead of samples or inhibitors. Blanks were also performed, in order to avoid background interference, containing buffer

instead of the enzyme. The inhibitory activity of the samples was measured by absorbance at 560 nm, as a result of the NBT transformation into the chromogen formazan formed by the superoxide radical. The results were expressed as a percentage of free radical scavenging capacity (RSC) and were calculated with Equation (1) considering blank substraction.

$$\text{RSC (\%)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}}] \times 100 \quad (1)$$

The *M. incana* extract activity on the xanthine oxidase enzyme was also evaluated by measuring the formation of uric acid from xanthine at 295 nm after 2 min. The reaction mixture contained the same components as those described previously in the xanthine/xanthine oxidase system, with the exception of NBT.

2.5. Enzyme Inhibition Activity

2.5.1. Acetylcholinesterase (AChE) Inhibition

The activity was measured using a 96-well microplate reader based on Ellman's method with some modifications [25]. The assay mixture contained 15 mM ATCI in Millipore water, 3 mM DTNB in buffer C (50 mM Tris-HCl, pH 8, 0.1 M NaCl, 0.02 M $MgCl_2{\cdot}6\ H_2O$), buffer (50 mM Tris-HCl, pH 8, 0.1% fetal bovine serum), and different concentrations of *M. incana* extract or reference inhibitor solved in buffer (50 mM Tris-HCl, pH 8). Then, AChE (0.22 U/L) was added to start the reaction. Controls were performed in order to obtain 100% activity, containing buffer instead of samples or inhibitors. Blanks were also performed, in order to avoid background interference, containing buffer instead of the enzyme. Absorbance was read 13 times every 13 s at 405 nm. Galantamine was used as a reference inhibitor. The results were expressed as a percentage of inhibition to the control wells and were calculated with Equation (2).

$$\text{Inhibition (\%)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}}] \times 100 \quad (2)$$

2.5.2. Monoamine Oxidase A (MAO-A) Inhibition

The assay was performed in a 96-well microplate using the technique previously described [25]. Each well contained *M. incana* extract or reference inhibitor (clorgyline) at different concentrations, chromogenic solution (0.8 mM vanillic acid, 417 mM 4-aminoantipyrine, and 4 U mL^{-1} horseradish peroxidase in potassium phosphate buffer pH 7.6), 3 mM tyramine, and 8 U/mL MAO-A. Controls were performed in order to obtain 100% activity, containing buffer instead of samples or inhibitors. Blanks were also performed, in order to avoid background interference, containing buffer instead of MAO-A. The absorbance was read at 490 nm every 5 min for 30 min. The results were calculated with Equation (2).

2.5.3. Tyrosinase (TYR) Inhibition

The assay was conducted in 96-well microplates using a microplate reader to measure absorbance at 475 nm using the described procedure [26]. *M. incana* extract at 10 µL or reference inhibitor (Kojic acid) at different concentrations, L-DOPA, phosphate buffer (pH 6.8), and tyrosinase were added to each well. Controls were performed in order to obtain 100% activity, containing buffer instead of samples or inhibitors. Blanks were also performed, in order to avoid background interference, containing buffer instead of the TYR. The results were calculated with Equation (2).

2.5.4. Lipase Inhibition

The activity was measured in 96-well microplates using previous protocols [27]. Each well contained *M. incana* extract or reference inhibitor (orlistat) at different concentrations and 2.5 mg/mL lipase, prepared in 100 mM Tris and 5 mM $CaCl_2$ buffer, pH 7.0. After 15 min preincubation, 20 µL of 10 mM pNPB solution was added to each well for another 15 min incubation at 37 °C.

Controls were performed in order to obtain 100% activity, containing buffer instead of samples or inhibitors. Blanks were also performed, in order to avoid background interference, containing buffer instead of lipase. Absorbance was read at 405 nm, and orlistat was used as a reference inhibitor. The results were calculated with Equation (2).

2.5.5. Inhibition of α-Glucosidase (α-GLU)

The capacity of *M. incana* extract to inhibit α-glucosidase was measured in a 96-well microplate reader based on the method previously described [27]. Each well contained a reaction mixture of *M. incana* extract or reference inhibitor (acarbose) at different concentrations and α-GLU (1.0 U mL^{-1}). After preincubation for 10 min, 3.0 mM pNPG (dissolved in 20 mM phosphate buffer, pH 6.9) was added to start the reaction and incubated at 37 °C for 20 min. Then, absorbance was measured at 405 nm. Controls were performed in order to obtain 100% activity, containing buffer instead of samples or inhibitors. Blanks were also performed, in order to avoid background interference, containing buffer instead of α-GLU. The results were calculated with Equation (2).

2.6. Statistical Analysis

The results of cytotoxicity tests were the mean ± standard deviation (SD) of four experiments in triplicate. One-way analysis of variance (ANOVA), followed by Bonferroni's t-test, was performed in order to estimate significant differences among groups. Data were reported as mean values ± standard deviation (SD), and differences among groups were significant at $p < 0.001$. The results of the antioxidant and enzyme inhibiting assays were obtained from at least the average of three independent experiments and were expressed as mean ± standard error (SEM). Data analyses were run with GraphPad Prism v.6 (GraphPad Software, San Diego, CA 92108, USA).

3. Results and Discussion

3.1. Cytotoxic Activity

Even though there has been significant progress in the fight against cancer, the discovery of bioactive natural products still plays an important role in the research and development of new anticancer agents to treat it effectively and with fewer adverse effects. In order to provide a scientific basis for the empiric use of *M. incana* as an anticancer agent, we tested the cytotoxic activities of hydroalcoholic extract of this species against two human cancer cell lines: MCF7 breast cancer cells and CaCo-2 colorectal adenocarcinoma cells. No change in viability was observed in MCF-7 cells treated with 0.0625-4 mg/mL of *M. incana* extract for 48 and 72 h. This result does not support the reported traditional use of *M. incana* for the treatment of breast cancer.

In contrast, as shown in Figure 1, the treatment of CaCo-2 with different concentrations of *M. incana* extract induced a moderate inhibitory effect on succinate dehydrogenase activity at both 48 and 72 h of exposure. The exerted cytotoxic activity was significant, starting from the concentration of 2 mg/mL at 48 h and from the concentration of 0.5 mg/mL at 72 h of treatment, where the inhibitory effects reached a value of 30% at 4 mg/mL. This distinct effect is probably due to the two different cell lines, which respond to the same treatment in different ways; indeed, MCF7 breast cancer cells are well known for their drug resistance capacity mediated by breast cancer resistance protein (BCRP) [28].

The cytotoxic effect detected on the CaCo-2 cell line by MTT assay was further investigated by the determination of LDH release. Figure 2 evidenced that non-necrotic cell death was associated with reduced viability at the lowest concentrations of the extract, allowing us to hypothesize the involvement of a different death pathway for both times of exposure. Conversely, at the highest concentration (4 mg/mL), we found a significant increase in LDH release suggesting a necrotic effect due to the high concentration of plant metabolites present in the extract (Figure 2).

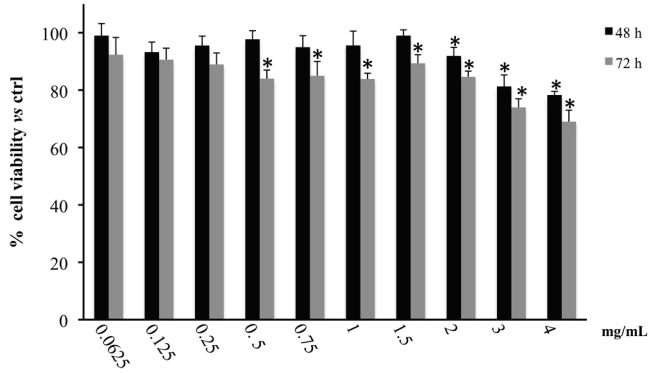

Figure 1. Cell viability in CaCo2 cells treated for 48 and 72 h with the hydroalcoholic extract of *M. incana* aerial parts evaluated by MTT assay. Values are the mean ± SD of four experiments in triplicate. * Significant vs. untreated control cells: $p < 0.001$.

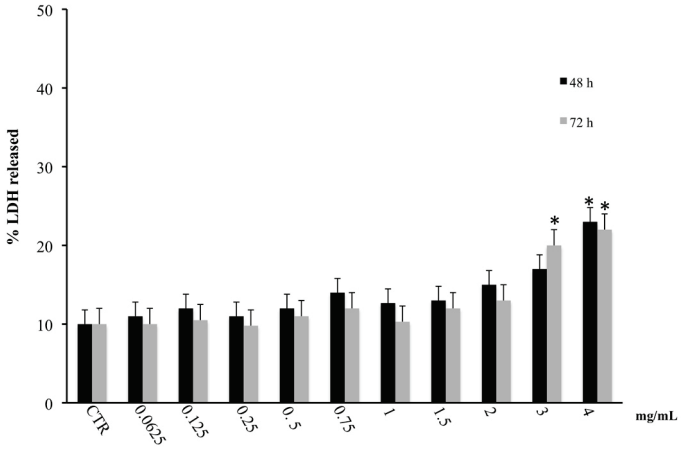

Figure 2. LDH released in CaCo2 cells untreated and treated for 48 and 72 h with the hydroalcoholic extract of *M. incana* aerial parts. Values are the mean ± SD of four experiments in triplicate. * Significant vs. untreated control cells: $p < 0.001$.

The potential cytotoxic activity of *M. incana* extract determined in the CaCo-2 cell line could be related to its ferrous ions' chelating properties, as demonstrated in our previous study [15]. In fact, some metals such as copper and iron have been shown to play a significant role in the rapid proliferation of cancer cells [29].

3.2. Antioxidant Activity

Matthiola incana extract showed the ability to scavenge superoxide radicals produced by a xanthine/xanthine oxidase reaction (Figure 3). This antioxidant activity was lower than the reference compound, gallic acid. The IC_{50} values were 2.38 and 0.45 μg/mL for *M. incana* and gallic acid, respectively.

Antioxidant properties of phenolic compounds are widely demonstrated [30]. Previous studies by the authors characterized the polyphenolic compounds contained in *M. incana* extract and showed the potential of this plant as a radical scavenger, reducing agent, or metal chelator [15]. However, this was the first time that this antioxidant potential was observed in a radical superoxide, that is, one of the physiological reactive oxygen species presented as the product of numerous enzymatic reactions.

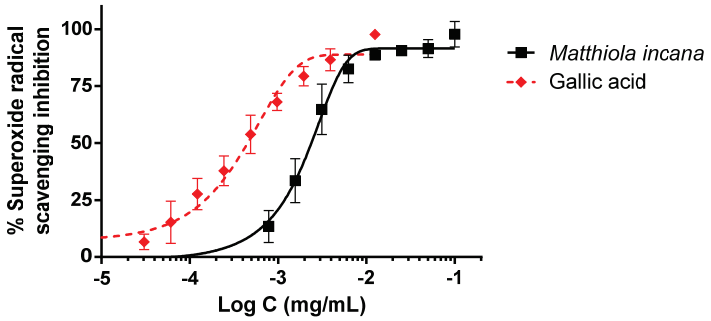

Figure 3. Antioxidant activity of the hydroalcoholic extract of *M. incana* aerial parts evaluated in the xanthine/xanthine oxidase system. IC_{50} values were calculated by non-linear regression. All concentrations were tested at least in triplicate, and each point represents mean ± SEM.

3.3. Inhibitory Activities on CNS Enzymes

Matthiola incana extract was able to inhibit CNS enzymes as MAO-A and TYR but not AChE. In the MAO-A inhibition assay, the extract reached complete enzyme inhibition but only at the highest doses (Figure 4A). The difference between the extract and clorgyline was obvious, as also confirmed by the IC_{50} values, 570.28 and 0.15 μg/mL for the extract and the reference inhibitor, respectively. However, the extract showed higher activity in the TYR inhibition assay, reaching almost 90% of inhibition and a very similar profile to the reference inhibitor, kojic acid (Figure 4B). The IC_{50} values were 25.21 and 3.52 μg/mL for the extract and the reference inhibitor, respectively. However, the extract was not able to inhibit AChE in any tested concentration.

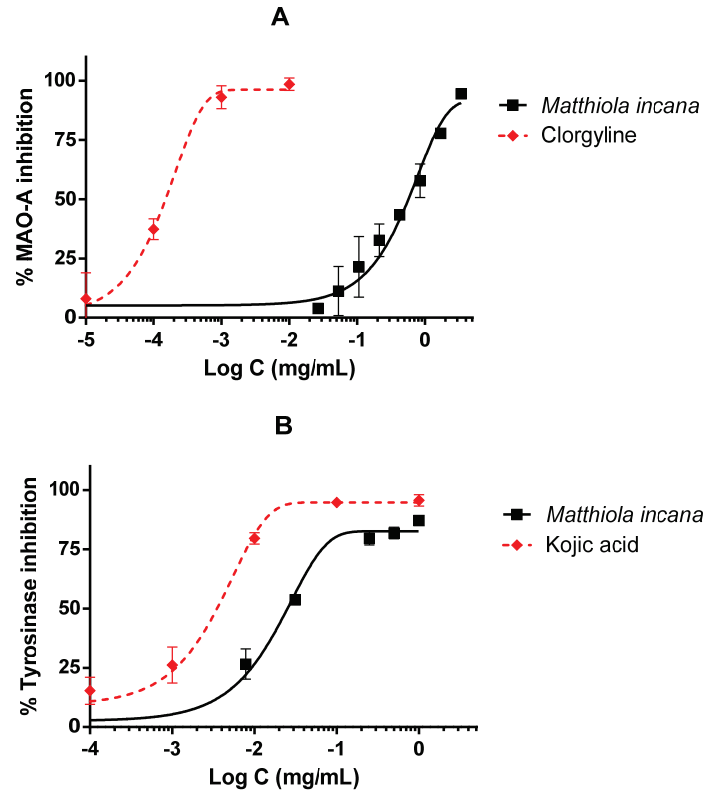

Figure 4. Activity of the hydroalcoholic extract of *M. incana* aerial parts in enzymes related to central nervous system (CNS) pathologies. IC_{50} values were calculated by non-linear regression. All concentrations were tested at least in triplicate, and each point represents mean ± SEM. (**A**): monoamine oxidase A (MAO-A) inhibition performed by *M. incana* extract and clorgyline as standard. (**B**): tyrosinase (TYR) inhibition by *M. incana* extract and kojic acid as standard.

This was the first time that these inhibitory properties on CNS enzymes were shown for this species. However, it is known that antioxidants and extracts with high polyphenol content could be involved in the prevention of diseases related to oxidative stress as neurodegenerative pathologies [31,32]. Synthetic MAO inhibitors have been used to treat depression or dementia but may have side effects due to the increase of biogenic amines in the blood. Kaempferol, a flavonoid contained in the extract, has already demonstrated its potential inhibitory effect on human MAO-A [33,34], which could explain the inhibition of this enzyme by the *M. incana* extract. TYR inhibition may represent a potential neuroprotective strategy preventing dopamine-induced neuronal damage, although TYR inhibitors are more often used in preventing skin pigmentation in dermatology [35]. Phenolic compounds present in this extract as kaempferol or luteolin inhibited TYR [36,37]. However, naringenin, another flavonoid contained in high concentration in the extract, exhibited significant anti-proliferative activity against B16F10 melanoma cells and enhanced TYR activity, suggesting its use as a natural tanning agent [38].

3.4. Inhibitory Activities on α-Glucosidase (α-GLU) and Lipase

Matthiola incana extract was also tested as an inhibitor of enzymes presented in the digestive tract, with the aim of investigating the potential use of this extract as an antidiabetic and/or anti-obesity herbal drug. The extract was able to inhibit around 50% of lipase activity at the highest tested concentration (Figure 5A). IC_{50} for the extract was 508.71 μg/mL, considerably higher than reference inhibitor, orlistat, whose IC_{50} was 0.70 μg/mL. Nonetheless, the inhibitory activity of *M. incana* was better in the α-GLU assay. As shown in Figure 5B, the extract inhibited the enzyme in a dose-dependent manner with a better response than the reference inhibitor acarbose. The inhibitory curve of the extract was slightly shifted to the left relative to acarbose; however, no significant differences were detected between acarbose and the extract (142.20 and 378.92 μg/mL for the *M. incana* extract and acarbose, respectively).

This was also the first time that this extract was shown to be able to inhibit enzymes involved in metabolic disorders such as lipase and α-GLU. Although inhibitory effects were detected in commercial glucosidases from *Saccharomyces cerevisiae*, this enzyme is widely used to screen glucosidase inhibitors with potential use and applications in human health; nevertheless, more studies will be performed in the future in order to establish the inhibitory activity on human glucosidases. Oxidative stress is also responsible for metabolic syndrome pathologies [39,40], and, for this reason, different antioxidants have been studied in this field. There is only one study in which *M. incana* seeds, rich in linolenic acid oil (55–65%), reduced cholesterol levels and increased omega-3 fatty acid levels in the plasma of rats [41]. However, the phytochemical composition cannot be compared to *M. incana* aerial parts, as this extract is particularly rich in polyphenols; among flavonoids detected in this extract, kaempferol, naringenin, and luteolin were found to inhibit α-GLU [32–46]. Kaempferol was also able to inhibit lipase [47]. Luteolin and naringenin have been demonstrated to protect against severe acute pancreatitis in mice by exerting anti-inflammatory and antioxidant effects and decreasing lipase activity [48,49]. The ability of the isolated flavonoids presented in the extract could explain the activity of the extract in these enzymes. Moreover, another study with kaempferol demonstrated the capability to decrease adipogenesis in the 3T3-L1 cell line and lipid accumulation in mature adipocytes [50].

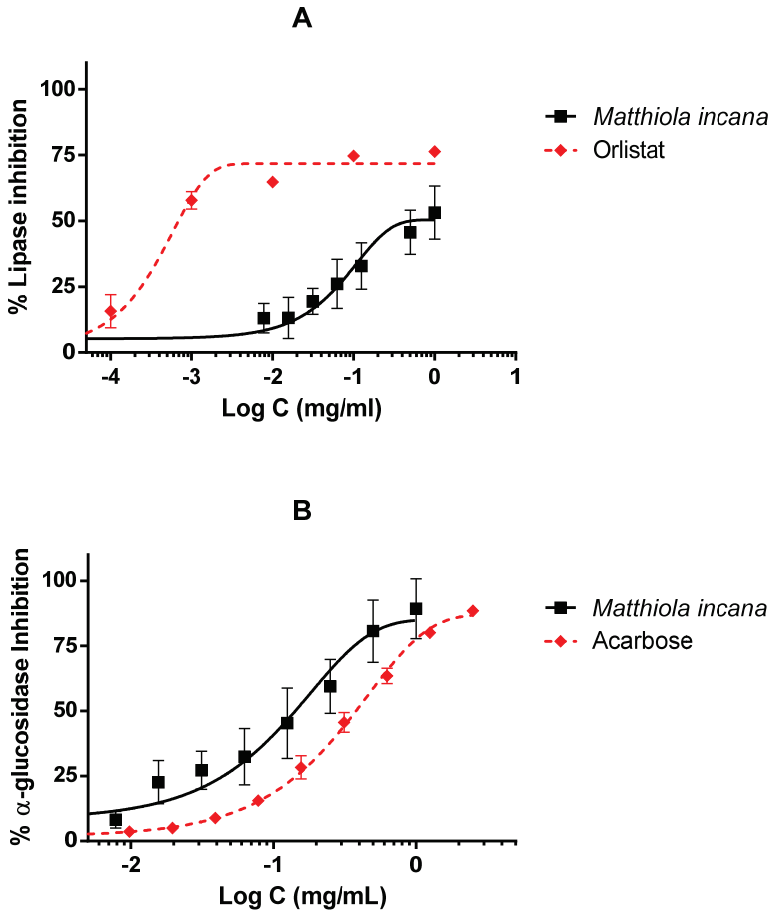

Figure 5. Activity of the hydroalcoholic extract of *M. incana* aerial parts on lipase (**A**) and α-glucosidase (**B**). IC_{50} values were calculated by non-linear regression. All concentrations were tested at least in triplicate, and each point represents mean ± SEM. A: Lipase inhibition performed by *M. incana* extract and orlistat as standard. B: α-glucosidase (α-GLU) inhibition by *M. incana* extract and acarbose as standard.

4. Conclusions

Our findings highlight that the hydroalcoholic extract of *M. incana* aerial parts displays moderate to low cytotoxicity vs. CaCo-2 cells and acts as a superoxide radical scavenger and enzymatic inhibitor of MAO-A, TYR, α-GLU, and lipase. The best results were found in the α-GLU assay, which paves the way for further studies aimed at evaluating the inhibitory effect against mammalian α-glucosidases and, thus, the potential use of *M. incana* extract in the management of postprandial hyperglycemia in type-2 diabetes.

The aerial parts of *Matthiola incana* represent an interesting source of bioactive compounds with antioxidant properties and the potential capability of preventing neurodegenerative or metabolic diseases through enzyme inhibition mechanisms. However, other investigations need to be carried out in living organisms in order to explore the bioactivities highlighted in this study and to identify the active compounds.

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