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Phytochemicals of Natural Products

Analysis and Biological Activities

Edited by
Dasha Mihaylova and Aneta Popova

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Editors

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Editorial

Phytochemicals of Natural Products: Analysis and Biological Activities

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The natural products of the plant kingdom, both terrestrial and marine, have been widely explored. Plant-based products have long been used in the prevention and treatment of various ailments. Many natural compounds have been reported to have a variety of interesting and significant biological activities (antioxidant, anti-inflammatory, antibacterial, antifungal, antiparasitic, analgesic, antidiabetic, anti-atherogenic, and antiproliferative). Therefore, researchers have paid special attention to the bioactive compounds synthesized by plants. Opportunities in the fields of functional ingredients and the treatment of non-communicable diseases (oxidative stress, diabetes, obesity, metabolic syndrome, etc.) have been widely explored. However, undeniably, many of the therapeutic properties of plants are yet to be discovered. The current Special Issue, "Phytochemicals of Natural Products: Analysis and Biological Activities", compiles 11 original research articles and 3 reviews focusing on the chemical profiling of plants, the beneficial properties of their essential oils, and the practical application of plant by-products, with their antimicrobial and antioxidant activities and specific volatile profile.

Phenolic compounds are a group of natural organic substances that are found in different morphological plant parts. They exhibit particularly strong antioxidant properties that protect the body from the damaging effects of free radicals. In the experiment conducted by Afonso et al. [1], it was stated that total phenol levels in hop cones were substantially affected by experimental factors such as plant vigor, foliar treatment, liming, cultivar, plot, and year. Agro-environmental variables make it difficult to recommend strategies to both farmers and researchers without a well-defined target for the further utilization of the product. The paper of Yin et al. [2] confirmed that metabolic differences between the medicinal and non-medicinal parts of plants exist, and they may not possess the same therapeutic effect, which warrants a strict regulation of harvest and market standards. Kolarević et al. [3] revealed the potential of in vitro leaves of blueberry as a source of total phenolics and flavonoids. The aqueous extracts of field-grown and in vitro leaves of blackberry and blueberry were screened, and the results show that they can be a good source of phenolic compounds and exhibit antioxidant properties, whereas callus cultures have potential for the production of specific phenolic compounds. Consumers have a growing interest in foods that offer high nutritional value and provide health benefits. Rehal et al. [4] proposed the utilization of tomato pomace (a by-product) for the preparation of ready-to-cook gluten-free snacks. The newly developed product helped to lower the oil uptake and enhanced the fiber, lycopene content, mineral, and antioxidant activity.

The antioxidant potential of plant sources has been widely studied for a long time. Yet, new knowledge is continually being added to that already published on the subject. Gentscheva et al. [5,6] studied the potential of extracts from *Sempervivum tectorum* L. and *Sambucus nigra* L. blossoms to be used for the preliminary prognosis of pharmaceutical applications due to their phenolic content and antioxidant potential. The abovementioned authors also stated the total element contents (Ca, Mg, Zn, Mn, and Fe) in leaves of *Sempervivum tectorum* L. They focused on the trace element content of freshly squeezed

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S. tectorum leaves as an essential plant characteristic. The established content of toxic elements in plants grown on different soils clearly showed pollution from urban soils and phosphate fertilizers. Furthermore, the authors presented a correlation between the total phenolic contents and the antioxidant activities of *S. tectorum* water–ethanolic extracts [7]. In addition, Bulgarian *S. nigra* samples (blossoms and leaves), collected from different regions, were screened and compared in terms of their phytochemicals and activities [5].

The team of Duong et al. [8] focused on Curcuma (turmeric) species, and the essential oils of Curcuma rhizomes, which have demonstrated promising pharmacological properties. They also confirmed that wide variation exists in the essential oil compositions from different geographical locations. The volatile profile of plants has always been an object of extensive study. The review of Tangpao et al. [9] provides information about the usefulness of volatile organic compounds from diverse types of basil essential oils and their biological activities, summarizing techniques for enhancing the efficacy of the extraction process. The study presented by Plabon et al. [10] showed that the peel of *Spondias mombin* is distinctive in terms of its volatile composition. The antimicrobial activity of the oil was analyzed by the disk diffusion method. The results in terms of the antifungal activities of the peel oil showed the highest zone of inhibition against *Aspergillus niger* and *Penicillium oxalicum*, pointing toward *S. mombin* as an alternative to synthetic bactericides and fungicides to be used in the agro-industries. Lafraxo et al. [11] promoted the potential of *Juniperus thurifera* essential oil as an alternative solution to antibiotics due to its activity against the fungal strains *C. albicans*, ATCC 10231, and *F. oxysporum*, MTCC 9913. Ismail et al. [12] tested different extracts of pomegranate, sugar apple, and eggplant peels applied as inhibitors of the aflatoxigenic maize fungus *A. flavus*. GC–MS phytochemical analysis of fruit peel extracts suggested that compounds such as α-kaurene, α-fenchene, p-allylphenol, octadecanoic acid, 3,5-dihydroxy phenol, hexestrol, xanthinin, and linoleic acid could be the active substances. Moreover, fruit peel waste extracts have been reported as a potential source of useful compounds.

A review article written by Panda and Duarte-Sierra [13] shows plants as a reservoir of phytochemicals, with several beneficial health properties. Panda and Duarte-Sierra pinpoint the importance of plant polyphenols, their role as antimicrobial agents, the mechanisms of biochemical methods, and the ways these methods may be used in enhancing the antimicrobial potency of plant polyphenols. Finally, Madrigal-Santillán et al. [14] evaluated the available scientific evidence of the beneficial properties of *Opuntia* spp., with reference to its protective effects against atherosclerotic cardiovascular disease, diabetes, and obesity, in addition to hepatoprotection, among other effects, outlining a direction for further investigations (in vitro, in vivo, and clinical) to confirm these properties.

Modern horticulture must turn its attention toward the supply of products with a variety of valuable metabolites and aromas. This has motivated researchers to study these compounds and facilitate the development of horticultural products. All of the above highly encourages both health professionals and researchers to expand their studies on the pharmacological and therapeutic effects of plants.

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

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Article

A Tomato Pomace Enriched Gluten-Free Ready-to-Cook Snack's Nutritional Profile, Quality, and Shelf Life Evaluation

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Abstract: Attempts were undertaken to design a quick ready-to-cook gluten-free snack utilizing finger millet and potato flour (50:50) as well as tomato pomace due to the restricted availability of gluten-free snack goods in the Indian market. The nutritional content of the food and its general acceptability, cooking characteristics, and storage stability were all tested. The addition of tomato pomace had a distinct influence on the product's color and hardness. Additionally, it resulted in a significant reduction in the amount of oil used, cooking loss, and frying time required. With a high acceptance level, the snack supplemented with 10% tomato pomace was determined to be the most optimal formulation. When the same substance was subjected to FTIR analysis, it was discovered that it retained all the important functional groups required for sustaining antioxidant activity. It also displayed high storage stability, a desirable overall acceptance score, and a very promising nutritional profile, all of which would benefit the product's end users.

Keywords: tomato pomace; by-product utilization; finger millet; gluten-free; potato flour; convenience food

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1. Introduction

Tomato (*Solanum lycopersicum*), touted to be the most important vegetable crop, is consumed both in its fresh and processed forms. Its production in India amounted to 21.18 million metric tons in 2021 [1]. Tomato processing generates 1.5 to 5% pomace, which is a waste product but is a storehouse of nutrition [2,3]. It is used as animal feed or is dumped in landfills [4], thus having both economic and environmental implications since additional costs for transportation are required. Therefore, recycling and reusing pomace can reduce tomato processing costs [5].

Majority of the world population consumes wheat in one form or the other but in recent years, increasing number of people are showing sensitivity towards the gluten present in wheat, rye, and barley, which causes health problems for them [6]. The symptoms include fatigue, weight loss, diarrhea, and depression [7]. Since there is no cure yet for celiac disease, the only option is to have a gluten-free diet. Any food that contains less than 20 ppm (or 20 mg/kg) of gluten is defined by United States Food and Drug Administration (FDA) as gluten free [8]. Furthermore, not all consumers who prefer gluten-free food have celiac disease. Many switch to it as a matter of personal preference, to meet specific dietary requirements, as fasting food, or just to have healthy food [9,10]. Additionally, there is a change in eating patterns and individual preferences, and experimentative palates have resulted in the creation of new markets for ready-to-cook foods that effectively reduce

drudgery, cooking time, and procurement of multiple ingredients while adding convenience and variety to the meal [11]. Consumers perceive readymade frozen foods as nourishing, healthy, and delicious [12]. In this scenario, snacks have now become an important part of any diet and biting into a healthy snack serves both the purposes of convenience and nutrition. Besides being gluten-free, the snack should serve the dual purpose of being able to be indulged in as well as provide nutrition for wider acceptance. However, the availability of convenient gluten-free traditional snacks is very limited in the Indian market, and even if available, these are expensive [13].

Finger millet (*Eleusine coracana* L.) is the most consumed small millet in India and accounts for 85% of all millet produced in India [14]. It has an excellent nutritional profile compared to commonly consumed cereals [15–17]. Its crude fiber and mineral contents are markedly higher than those of wheat (1.2% fiber and 1.5% minerals) and rice (0.2% fiber and 0.6% minerals); its protein is relatively better balanced; and it contains more lysine, threonine, and valine than other millet. It has the highest calcium content among all cereals (344 mg/100 g), which is almost three times more than milk and tenfold higher than brown rice, wheat, or maize [17]. The total dietary fiber content of finger millet grain (19.1%) is reported to be the highest compared to that of many other cereal grains (12.1%, 3.7%, 12.8%, and 11.8%, respectively, for wheat, rice, maize, and sorghum) [16].

Despite the ample availability of tomato pomace as well as a healthy gluten-free alternative grain, there are not many products on the market to meet the demand for convenient gluten-free snacks due to the lack of optimized methods for their preparation. The utilization of tomato pomace in food products is challenging, owing to its highly perishable nature, acidity, and the lack of knowledge of how it affects the quality attributes of the prepared product.

The present study was hence undertaken with the purpose of developing a ready-to-cook gluten-free (RTC-GF) snack using finger millet and tomato pomace that has good acceptability and palatability and to study the nutritional traits and shelf-life of the product.

2. Materials and Methods

Fresh tomato pomace (variety: *Punjab Ratta*), devoid of any foul odor or taste, was obtained from the Food Industry and Business Incubation Centre, Punjab Agricultural University, Ludhiana. Immediately after procurement, it was dried in a tray dryer at 50 °C for 48 h and then milled in the mixer grinder (Inalsa Inox 1000 model) and sieved through 40 mm sieve to get a fine tomato pomace powder (TPP). The powder was packed in polyethylene bags and stored in a refrigerator at 4 ± 2 °C till further use.

More than 1500 germ plasm lines of finger millet were acquired by PAU, Ludhiana from NBPGR, New Delhi, due to the feasibility of its profitable cultivation in Punjab. After initial screening, a high yielding, stable, and promising line, IC0475677, was shortlisted for further testing. Seeds from this line were procured from the university's Department of Plant Breeding and Genetics, thoroughly cleaned and washed, and then dried at 60 ± 2 °C for 6 h in a tray dryer. They were milled to get finger millet flour (FMF) using a Laboratory Mill 3303 (Perten Instruments), sieved through a 40 mm sieve, packed in polyethylene bags and stored in a refrigerator at 4 ± 2 °C till further use.

Potato flour (PF) was prepared by the standard procedure, where the potatoes are washed, peeled, diced, boiled, dried in a cabinet drier at 50 °C, ground, sieved, and packed in airtight containers [18]. Fresh ginger, garlic, green chilies, and coriander, along with salt, spices, and refined soybean oil, were procured from the local market.

2.1. Product Preparation

In the initial trials, FMF was used either as a powder (after roasting in an open pan with continuous stirring till a toasty aroma emanated) or as a cooked paste (by cooking the flour in four times water to obtain a thick gruel here called finger millet paste (FMP)). It was found that the addition of finger millet as a cooked paste gives a product with better handling, binding, pliability, and texture, and the paste was hence chosen to be added in

the final formulation. Sensory experiments showed that the finger millet paste (FMP) can be substituted with up to 50% of PF to get the best sensory scores [19]. A mixture was prepared containing 30% finely chopped onion, 15% crushed ginger, 10% crushed garlic, 10% green chili, 15% green coriander, 15% salt and 5% garam masala spice mix and it was added in each formulation. The base formulation of the RTC-GF snack contained FMP and PF in equal proportions. This represented the control sample (T1) in which there was no addition of TPP, but the spice mixture was added. Further experiments were done to standardize the addition of TPP to ascertain its maximum utilization without impairing the quality of the product. The TPP was substituted in the mix at 0, 5, 10, 15, 20, and 25% levels to obtain sample T1, T2, T3, T4, T5, and T6, respectively. The various treatments and their formulations are tabulated under Table 1.

Table 1. Formulations of the various treatments for one kg mix of RTC-GF snack.

Treatments (% of TPP)	TPP(g)	FMP + PF (1:1) (g)	Spice Mixture(g)
T1 (0%)	0	950	50
T2 (5%)	47.5	902.5	50
T3 (10%)	95.0	855.0	50
T4 (15%)	142.5	807.5	50
T5 (20%)	190.0	760.0	50
T6 (25%)	237.5	712.5	50

The product was prepared by following the procedure as given by Rehal et al. [19] and the pictorial representation of the same is given in Figure 1. The RTC-GF snack was analyzed for its various cooking attributes and sensory evaluation (as per details given in the Sensory Evaluation section). The formulation which was found most acceptable by the panelists and which would enable the maximum utilization of TPP was selected for conducting storage studies and nutritional status. The most acceptable formulation was then prepared, flash-fried, cooled, and packed in LDPE and HDPE pouches, and stored at −20 °C. These were analyzed for various quality characteristics like free fatty acids, peroxide value, and sensory evaluation after 0, 15, 30, 45, 60, 75, and 90 days of frozen storage by finish-frying the product immediately after taking it out of frozen storage as conducted by Rehal et al. [19].

2.2. Physico-Chemical Analysis

The moisture, protein, fat, ash, and fiber content of the raw materials as well as of the RTC-GF snack were determined as per the method prescribed by AOAC [20]. Total carbohydrates were estimated by the difference method, as in Yadav et al. [21]. The energy content in kcal/100 g was calculated as per Sehgal et al. [11]. For mineral estimation, one gram of sample was digested in a combination of nitric acid and perchloric acid (3:1), made up to a volume of 50 mL using deionized water. The minerals present in it were then estimated by plasma atomic emission spectrometry (Thermo Scientific, Waltham, MA, USA), as shown by Kaur et al. [22], where the samples were digested using a di-acid mixture, made up to the required volume with deionized water, filtered, and then further used for estimation. The amino acid analysis was carried out according to the method given by Musaalbakri et al. [23]. The estimation was done using HPLC, undertaking the hydrolysis of the protein with 6 M HCL containing 0.1 per cent phenol 110 °C for 24 h. Methionine was determined after pre-hydrolysis with performic acid oxidation before HPLC analysis, whereas tryptophan was determined by alkaline hydrolysis. Amino acids were expressed as g/100 g.

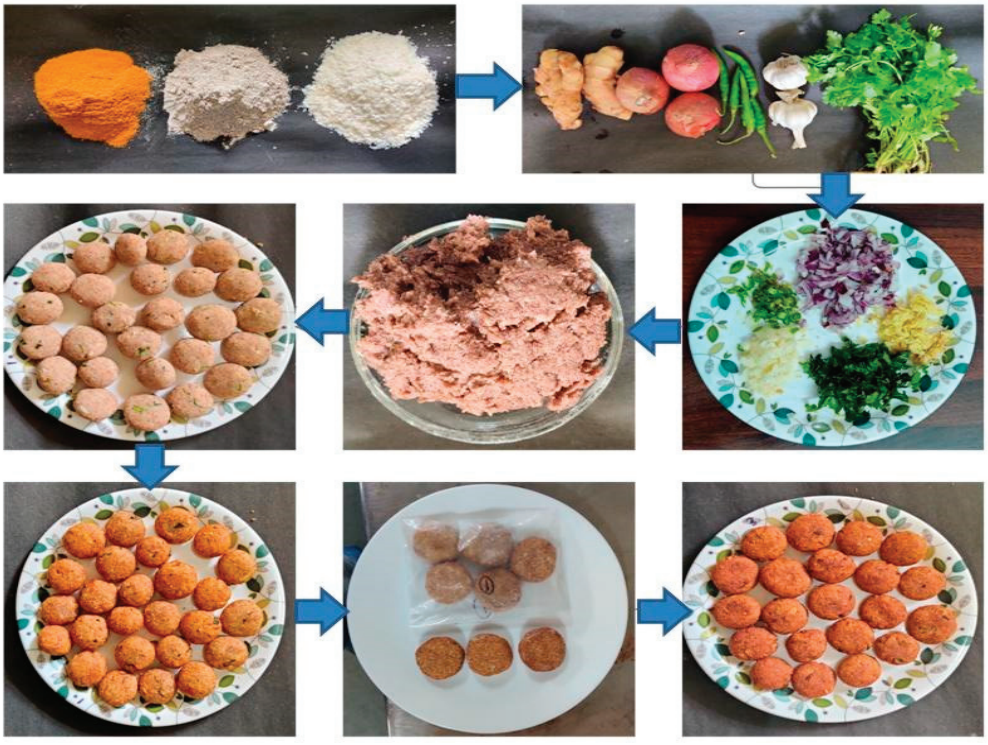


Figure 1. Process for Preparation of RTC-GF Snack.

The samples were analyzed for fatty acid composition by gas liquid chromatograph (model 7820A series, Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector with a CP-Sil 88 (25 m × 0.25 mm × 0.20 mm) FAME column. The temperature of the oven, detector, and injector were maintained at 210, 240, and 230 °C, respectively. Two µL of the sample were injected at a split rate of 10:1. Individual fatty acids were expressed as a percentage of total fatty acids, according to Hassanien et al. [24].

The color of the RTC-GF was determined in terms of L*, a*, and b* values where L* measured lightness ranging from black (L = 0) to white (L = 100), a* measured red (+) or green (-), and b* measured yellow (-) or blue (-) using a Minolta spectrophotometer colorimeter (ModelCM-508d). Hue angle (h°) and chroma (C) were measured as per Singh et al. [25].

2.3. Phytochemical Analysis

Lycopene was extracted and quantified by following the methods given by Ranganna [26] and Rodriguez-Amaya and Kimura [27]. The phenolic compounds were extracted by refluxing 50 mL of 80% *v*/*v* aqueous ethanol for 3 h at 40 °C and then total phenolic content (TPC) was determined as per the procedure given by Tapia-Salazar et al. [28]. The total flavonoids content (TFC) was determined by the aluminum chloride colorimetric method given by Zhishen et al. [29] and the results were expressed as quercetin equivalent (QE) mg/100 g db.

2.4. Antioxidant Analysis

The antioxidant activity of the samples was evaluated by preparing the extract of the samples. The weighed sample (one gram) was refluxed twice with 80% acidified methanol for 3 h. The pooled extracts were centrifuged at 1600× *g* for 10 min (Sorvall ST 16R, Thermo

Fischer Scientific, Bremen, Germany), with the volume made up with aqueous methanol, and these extracts were stored in amber bottles at 4 ± 1 °C till further analysis.

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was measured by using the method given by Herrara-Balandrano et al. [30]. The ferric reducing antioxidant power (FRAP) assay was performed as per the method given by Silva et al. [31] and the results are given as µmol ferrous sulphate equivalent (FSE)/100 g db. The 2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging activity was determined according to the method of Udeh et al. [32] and the results were expressed as µmol Trolox equivalent (TE)/100 g. The ability to chelate ferrous ions was determined to elucidate the metal chelating activity (MCA) by following the method detailed by Jayawardena et al. [33] and the MCA was reported in percentage (%).

2.5. Cooking Analysis

Cooking loss (%) was determined by taking the weight difference between raw and fried snacks. The frying time (secs) was noted as the time needed for the product to achieve a desirable reddish-brown color associated with fried foods. The oil uptake (%) was calculated by estimating the initial and final fat content and mass, before and after frying, of the product and computing the values using the equation given by Garmakhany et al. [34]. The texture profile analysis was carried out using a TA-XT texture analyzer (Stable Micro Systems Model TA-HDi, Surrey, UK) with an SMS P/5 probe to evaluate the crust hardness, where the hardness value is taken from where the peak force occurs during the first compression [35].

2.6. Sensory Evaluation

The evaluation of the sensory characteristics of the RTC snack were carried out through a semi-trained panel aged 24–58 years on a 9-point hedonic scale. The samples were coded (using different alphabet combinations) before being presented, and water was provided to rinse the mouth in between different samples. The panel scored the product for appearance, taste, flavor, texture, and overall acceptability.

2.7. Fourier-Transform Infrared (FTIR) Spectroscopy

Spectra of the finished product were taken on FTIR-ATR (Thermo Nicolet 6700 FTIR) spectrometer with attenuated total reflection (ATR) mode. A pinch of powdered sample was placed on the FTIR sample holder, and the spectrum was taken in the mid infra-red (IR) region (600–4000 cm^{-1}) [36].

2.8. Storage Studies

Free fatty acids (FFA%) such as oleic acid, peroxide value (PV), and moisture content were determined following standard protocols [20] and sensory studies of the optimized product (by the method given under sensory evaluation) were carried out every 15 days.

2.9. Statistical Analysis

The data were expressed as mean ± standard deviation (SD) (ten replicates for sensory analysis, three for others). The results were then subjected to analysis of variance (ANOVA) followed by Duncan multiple comparison test with $p \leq 0.05$ significance level using SPSS 18.0 statistical software.

3. Results

3.1. Physico-Chemical, Phytochemical, and Antioxidant Analysis

The proximate composition of the major raw materials (Table 2) shows that TPP has the highest amount of protein, followed by FMF and PF. A similar trend was observed for fat, ash, and crude fiber content of the raw materials whereas the reverse trend was observed for total carbohydrate content, with potato flour having the highest and TPP having the lowest carbohydrate content.

Table 2. Physico-chemical and phytochemical composition of major raw materials.

Parameter	Finger Millet Flour	Tomato Pomace Powder	Potato Flour
Moisture content (%)	8.56 ± 1.02 ^{a}	5.96 ± 1.12 ^{b}	6.08 ± 0.91 ^{ab}
Protein (%)	8.10 ± 0.90 ^{b}	14.95 ± 0.80 ^{a}	2.84 ± 1.01 ^{c}
Fat (%)	1.86 ± 0.18 ^{b}	8.52 ± 0.32 ^{a}	0.11 ± 0.11 ^{c}
Ash (%)	2.47 ± 0.21 ^{b}	4.27 ± 0.22 ^{a}	1.89 ± 0.15 ^{c}
Total carbohydrates (%)	79.01 ± 0.20 ^{b}	66.3 ± 0.21 ^{c}	89.09 ± 0.36 ^{a}
Crude fiber (%)	3.61 ± 0.87 ^{b}	39.45 ± 0.86 ^{a}	1.80 ± 0.73 ^{c}
TPC (mg GAE/100 g)	320 ± 1.10 ^{a}	179.67 ± 0.92 ^{b}	42.47 ± 0.87 ^{c}
TFC (mg QE/100 g)	56.11 ± 0.51 ^{b}	68.77 ± 0.57 ^{a}	6.20 ± 0.09 ^{c}
DPPH radical scavenging activity (%)	70.08 ± 0.3 ^{a}	52.41 ± 0.40 ^{b}	18.70 ± 0.32 ^{c}
FRAP (µmol FSE/100 g	2231.19 ± 15.6 ^{a}	1129.03 ± 7.9 ^{b}	584.15 ± 4.2 ^{c}
ABTS (µmol TE/100 g	913.01 ± 7.2 ^{a}	609.26 ± 4.0 ^{b}	33.19 ± 0.26 ^{c}
MCA (%)	58.25 ± 0.47 ^{a}	41.98 ± 0.36 ^{b}	18.73 ± 0.21 ^{c}
Calcium (mg/100 g)	274.3 ± 0.02 ^{a}	76.4 ± 0.01 ^{b}	67.2 ± 0.02 ^{c}
Phosphorus (mg/100 g)	295.7 ± 0.03 ^{a}	219.7 ± 0.02 ^{b}	59.30 ± 0.02 ^{c}
Magnesium (mg/100 g)	119.1 ± 0.01 ^{b}	126.7 ± 0.04 ^{a}	19.43 ± 0.01 ^{c}
Sodium (mg/100 g)	35.9 ± 0.01 ^{c}	129.1 ± 0.03 ^{a}	40.12 ± 0.03 ^{b}
Potassium (mg/100 g)	257.6 ± 0.02 ^{c}	1011.5 ± 0.04 ^{a}	532.6 ± 0.03 ^{b}
Iron (mg/100 g)	3.61 ± 0.03 ^{b}	9.26 ± 0.02 ^{a}	2.79 ± 0.01 ^{c}
Zinc (mg/100 g)	2.55 ± 0.04 ^{b}	3.46 ± 0.03 ^{a}	1.36 ± 0.02 ^{c}

Values are mean ± SD, n = 3; values within a row with different superscripts are significantly different ($p \leq 0.05$).

FMF showed a total phenolic content (TPC) of 320 mg GAE/100 g, which is significantly higher than that of TPP (179.67 mg GAE/100 g) and PF (42.47 mg GAE/100 g). The DPPH radical scavenging activity also followed a similar trend. Amongst minerals, calcium and phosphorus were found in higher amounts in FMF whereas magnesium, sodium, potassium, iron, and zinc were more prominent in TPP. Potato flour had significantly lower amounts of the estimated minerals amongst the raw materials.

Statistical analysis shows that the addition of TPP has a defined effect on the color values of the RTC-GF snacks (Figure 2). The control sample reported the highest L* values which decreased successively with the increase in percentage of TPP. It was observed that the supplementation beyond 15% had no significant effect on the L* values. The a* values, which depict the redness of the product, show a significant difference ($p \leq 0.05$) with a gradual increase. The addition of tomato pomace resulted in the decreased yellowness of the RTC-GF snack, which is reflected by lower b* values but the supplementation beyond 15% exhibited no significant difference ($p \leq 0.05$) in the b values. The hue angle ranged from 73.29° to 33.47° for T1 and T5, respectively, while the chroma values ranged from 22.40 to 18.53, where T3, T4, and T5 were not significantly different from each other ($p \leq 0.05$).

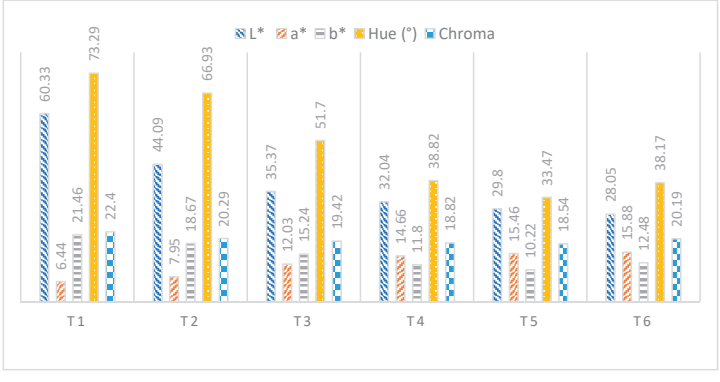


Figure 2. Color Scores of Ready-to-cook Gluten-free Snacks.

3.2. Cooking Analysis

The frying time of the RTC-GF snack showed a successive decrease from 42.41 s for sample T1, which contained no TPP, to 30.18 s in sample T5, which had 25% TPP. The hardness value of the control sample without TPP (T1) was 34.88 N which falls to 22.54 N due to the addition of 5% TPP (T2) but showed a continuous increase up to 36.6 N with an increase in the supplementation of TPP up to 25% (Figure 3a). Moreover, T1, which was made without the addition of TPP, exhibited the highest cooking loss (15.04%) and oil uptake (13.99%) amongst all the samples. These parameters showed a decrease in the samples as the concentration of TPP increased in T2 to T6 from 5 to 25% (Figure 3b). The oil uptake was found to be 7.42% while the cooking loss was as less as 6.73% in sample T6.

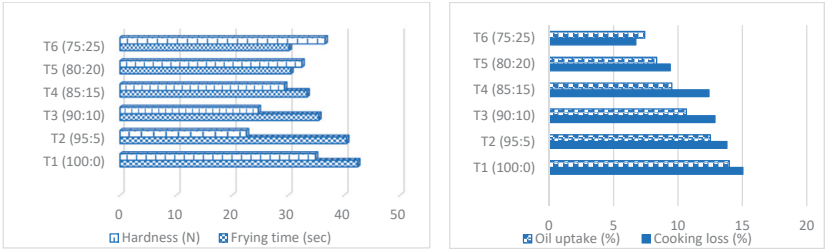


Figure 3. Cooking analysis: (**a**) Effect of addition of tomato pomace powder on the frying time and hardness of ready-to-cook gluten-free snack; (**b**) Effect of addition of tomato pomace powder on the oil uptake and cooking loss of ready-to-cook gluten-free snack.

3.3. Sensory Analysis

Figure 4 depicts the results of the sensory analysis of the RTC-GF snack, from where it is evident that the supplementation with TPP up to 25% has a profound effect on all the sensory parameters of the snack. In the case of taste, the scores showed a significant increase with up to 10% supplementation but beyond 20%, there was a significant decline in the scores for taste. A non-significant effect was observed for flavor at 0%, 5%, and 20% supplementation levels. There was a significant difference in 10% and 25% supplementation with TPP with regard to the flavor scores, with 10% obtaining the maximum score in all. The texture scores increased successively as the supplementation of TPP increased up to 10%, after which a significant decrease in the scores was observed with the increase in the levels of TPP. The snack with 10% of tomato pomace showed maximum overall acceptability due to the highest scores in appearance, taste, and texture, which were significantly higher than any other levels of supplementation. However, the snack having up to 20% supplementation level with tomato pomace also exhibited average sensorial attributes, but beyond that level, the overall acceptability scores declined.

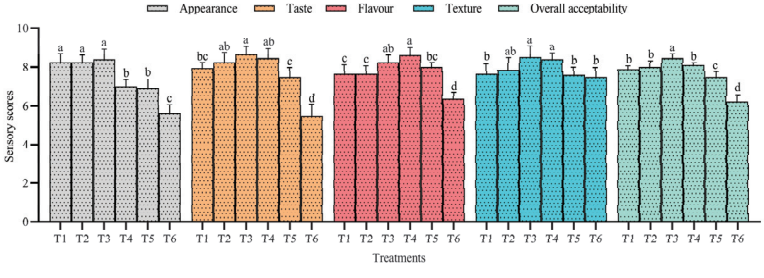
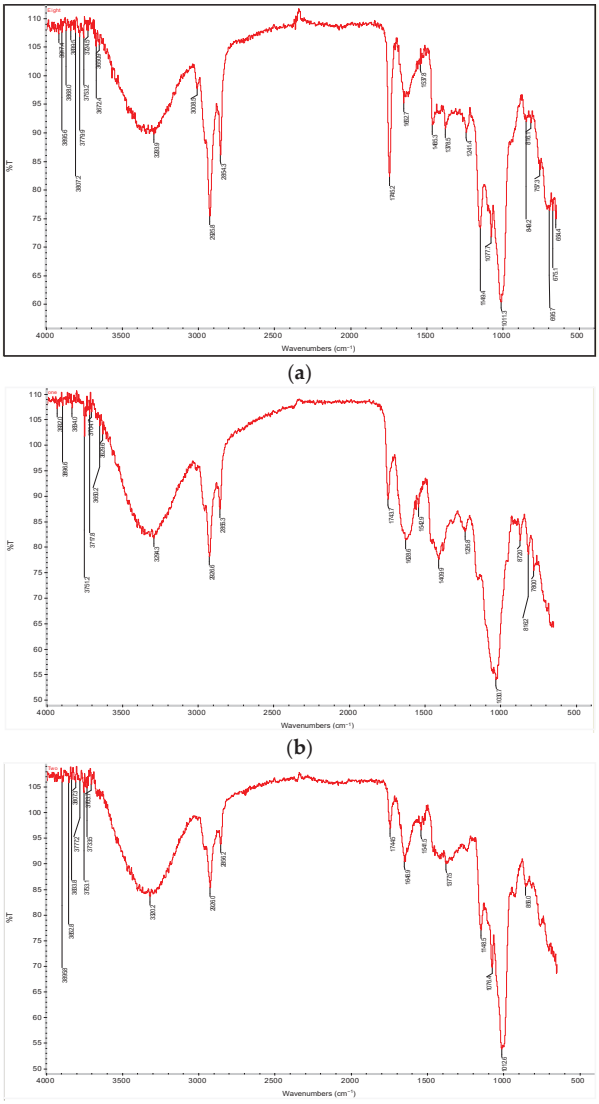


Figure 4. Sensory scores of the various formulations of ready-to-cook gluten-free snack. (Values are mean ± SD, n = 10; values within an attribute with different superscripts are significantly different ($p \leq 0.05$)).

3.4. Fourier-Transform Infrared (FTIR) Spectroscopy

Figure 5a–c presents the FTIR spectra of the finished RTC-GF snack, TPP, and FMF. Dominant peaks were observed at 2925 and 2855 cm^{-1} in all three spectra. The stretching areas were observed at 1376–1378 cm^{-1} in the FMF as well as RTC-GF snack. Absorption bands were observed at 1011.3, 1030.7, and 1012.6 cm^{-1} in the RTC-GF snack, TPP, and FMF, respectively. Similarly, peaks were also observed at a wavelength of 1744–1745 cm^{-1} as well as 2854–2856 cm^{-1} in all three spectra. The presence of absorption bands in the wavelength of 3290 to 3320 cm^{-1} were also found to be present in all the three commodities, *viz*., FMF, TPP, and RTC-GF (T3).



(**a**)

(**b**)

(**c**)

Figure 5. (**a**) FTIR spectrum of finished fried ready-to-cook gluten-free (T3) snack. (**b**) FTIR spectrum of tomato pomace powder. (**c**) FTIR spectrum of finger millet flour.

3.5. Nutritional Profile of the Product

The nutritional profile of the control RTC-GF snack containing no TPP (T1) and having 10% TPP (T3) is tabulated under Table 3. The enriched product provides a protein, fat, carbohydrate, and crude fiber content of 6.9%, 11.48%, 34.52%, and 5.2%, respectively, along with 248.2 kcal/100 g energy. The energy content of T3 is significantly less than the T1 sample containing no TPP. The TPC content is 186 mg GAE/100 g in T3 which was significantly higher than T1 (151.2 mg GAE/100 g). Similarly, the TFC also exhibited similar trend. The T3 had lycopene content of 5.08 mg/100 g while this was not detected in the T1 sample. The antioxidant activity as estimated by DPPH radical scavenging activity, FRAP, ABTS, and MCA for the RTC-GF snack shows significantly enhanced activity in the T3 sample as compared to T1. Potassium is the most abundant mineral present in the T3 RTC-GF snack followed by phosphorus, calcium, magnesium, sodium, iron, and zinc whereas T1 had amounts of phosphorus (206 mg/100 g) significantly higher than T3. Oleic acid is the most dominant fatty acid (52.1%) in T3, followed by linoleic acid (24.2%) and palmitic acid (22.8%), while T1 contains significantly higher amounts of palmitic acid as well as stearic acid. Amongst the essential amino acids, tryptophan is the highest (5.6%), followed by valine, phenyl alanine, isoleucine, and others in T3. It was observed that except for valine and leucine all other essential amino acids were present in significantly higher amounts in the T3 sample as compared to T1. The ω6/ω3 ratio was less for the T3 sample.

Table 3. Nutritional profile of ready-to-cook gluten-free snack (T1 (control) and T3).

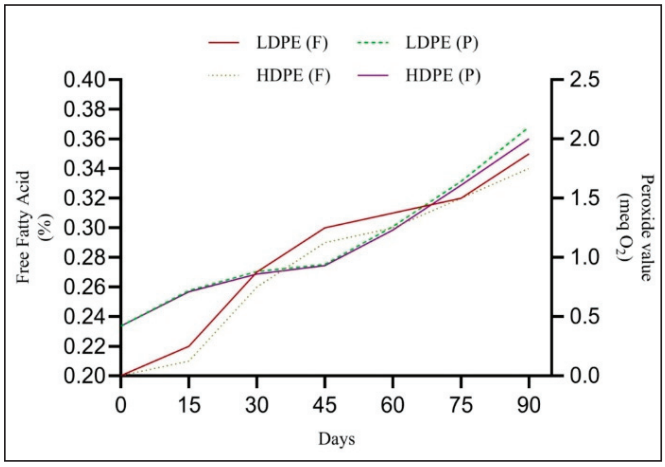
Parameter	T1	T3	Parameter	T1	T3
Moisture content (%)	40.5 ± 0.60 ^{b}	43.4 ± 2.6 ^{a}		Fatty acids (%)	
Protein (%)	5.01 ± 0.06 ^{b}	6.9 ± 0.16 ^{a}	Oleic acid	55.2 ± 0.82 ^{a}	52.1 ± 0.69 ^{b}
Fat (%)	17.45 ± 0.26 ^{a}	11.48 ± 0.70 ^{b}	Linoleic acid	25.6 ± 0.39 ^{a}	24.2 ± 0.29 ^{b}
Ash (%)	2.07 ± 0.27 ^{b}	3.7 ± 0.22 ^{a}	Linolenic acid	2.3 ± 0.02 ^{b}	3.4 ± 0.38 ^{a}
T. carbohydrates (%)	32.80 ± 0.51 ^{a}	34.52 ± 1.88 ^{a}	Palmitic acid	25.9 ± 0.37 ^{a}	22.8 ± 0.04 ^{b}
Crude fiber (%)	2.17 ± 0.25 ^{b}	5.2 ± 0.11 ^{a}	Stearic acid	6.2 ± 0.12 ^{a}	4.1 ± 0.52 ^{b}
TPC (mg GAE/100 g)	151.2 ± 2.01 ^{b}	186 ± 9.1 ^{a}	ω6/ω3	12.43: 1	7.1:1
TFC (mg QE/100 g)	31.06 ± 0.27 ^{b}	36.79 ± 0.30 ^{a}			
Lycopene (mg/100 g)	-	5.08 ± 0.09			
DPPH (%)	33.7 ± 0.64 ^{b}	46.7 ± 2.1 ^{a}			
FRAP (μmol FSE/100 g	1427.63 ± 9.9 ^{b}	1442.78 ± 10.11 ^{a}			
ABTS (μmol TE/100 g	537.11 ± 4.1 ^{b}	584.39 ± 4.4 ^{a}			
MCA (%)	40.70 ± 0.30 ^{b}	41.48 ± 0.34 ^{a}		Essential AAS (%)	
	Minerals		Valine	5.0 ± 0.10 ^{a}	5.2 ± 0.11 ^{a}
Calcium (mg/100 g)	152.6 ± 2.59 ^{b}	160 ± 1.01 ^{a}	Leucine	3.6 ± 0.08 ^{a}	3.5 ± 0.06 ^{a}
Phosphorus (mg/100 g)	206 ± 3.29 ^{a}	187 ± 1.60 ^{b}	Isoleucine	3.0 ± 0.06 ^{b}	4.53 ± 0.07 ^{a}
Magnesium (mg/100 g)	71.3 ± 0.78 ^{b}	89 ± 0.91 ^{a}	Threonine	3.4 ± 0.07 ^{b}	4.3 ± 0.06 ^{a}
Sodium (mg/100 g)	33.2 ± 0.53 ^{b}	40 ± 0.59 ^{a}	Methionine	2.9 ± 0.05 ^{b}	3.1 ± 0.04 ^{a}
Potassium (mg/100 g)	356.2 ± 5.69 ^{b}	407 ± 6.02 ^{a}	Lysine	3.1 ± 0.08 ^{b}	4.2 ± 0.06 ^{a}
Iron (mg/100 g)	2.79 ± 0.03 ^{b}	3.3 ± 0.06 ^{a}	Phenyl alanine	4.0 ± 0.12 ^{b}	5.1 ± 0.08 ^{a}
Zinc (mg/100 g)	1.82 ± 0.02 ^{b}	2.1 ± 0.03 ^{a}	Histidine	2.05 ± 0.03 ^{b}	2.2 ± 0.03 ^{a}
Energy (kcal/100 g)	308.29 ± 4.2 ^{a}	248.2 ± 5.3 ^{b}	Tryptophan	2.4 ± 0.04 ^{b}	5.6 ± 0.10 ^{a}

Values are mean ± SD, n = 3; values within a row with different superscripts are significantly different ($p \leq 0.05$).

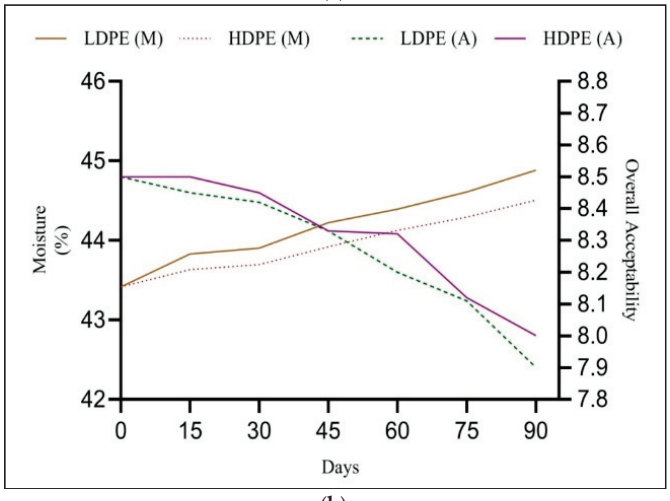
3.6. Storage Studies

Figure 6 shows the effect of frozen conditions on the RTC-GF snack. It is evident from Figure 6a that the free fatty acid content and peroxide value showed a steady increase in both the packaging materials but more so when the product was stored in LDPE than in HDPE pouches. The fresh samples had a free fatty acid content of 0.952% and the peroxide value was 0.42 meqO_2. The free fatty acid concentration increased to 1.197% for the snack in LDPE pouches and 1.175% in HDPE pouches after 90 days of storage. For the same time period, the peroxide value of the sample increased to 2.0 meqO2 in HDPE and 2.01 in LDPE pouches. Figure 6b represents the moisture content and overall acceptability of the

RTC-GF snack during its storage. The fresh product had a moisture content of 44.882%. It is evident that the moisture content of the stored product increased to 43.414% in LDPE and 44.505% in HDPE pouches after 90 days of storage. Regarding the sensory analysis of the product during storage, the overall acceptability scores decreased from 8.5 for fresh product to 7.9 for the snacks in LDPE pouches and 8.0 for the product in HDPE pouches on the 90th day of the storage study.



(**a**)



(**b**)

Figure 6. Storage studies: (**a**) Effect of packaging material on the free fatty acid content and peroxide value of stored ready-to-cook gluten-free (T3) snack; (**b**) Effect of packaging material on the moisture content(M) and overall acceptability(A) of stored ready-to-cook gluten-free (T3) snack.

4. Discussion

Of all the raw materials, tomato pomace powder (TPP) contained the highest fat, ash, and crude fiber content. The total phenolic content (TPC) was maximum for finger millet flour (FMF), followed by TPP and potato flour (PF) while TPP had the maximum total flavonoid content (TFC). The same pattern was observed for the DPPH radical scavenging activity. TPP retains its bioactive potential even after the tomatoes have undergone processing [3,37]. TPP also has maximum content of iron and zinc, while FMF is a rich source

of minerals, especially calcium, phosphorus, potassium, and magnesium. An estimated 17.3% of the world population is at risk of inadequate zinc intake [38] which is important for the immune system and metabolic activities. A combination of these raw materials in product development will help to meet the requirements of these micronutrients, which have established implications in various metabolic processes and health benefits, in diet.

The addition of TPP had a defined effect on the color values of the RTC-GF snack (Figure 2). The a* values, which depict the redness of the product, increase significantly ($p \leq 0.05$) with the gradual addition of TP due to the presence of lycopene in it. A higher a* value hinders the acceptability of a sample by consumers [39,40]. Hue angle is used for perception of color while chroma indicates the degree of departure of a color from a grey of the same lightness [25]. The increase in L* values with frying may be attributed to the darkening resulting from Maillard's reaction products [41].

The frying time decreased with the increase in the TPP levels in the snack (Figure 3a). This might be attributed to the fact that the crust achieved a brown color faster due to the higher lycopene content, which in turn is due to an increased percentage of TPP, which might affect one's perception of the quick cooking time, due to the darkening of the crust. A similar trend was also observed for the cooking loss per cent and oil up-take per cent of the snack (Figure 3b). This could be due to the increased moisture binding capacity of TPP, attributed to its fiber content, as evident by the highest cooking loss in T1 with no TPP. Oil uptake by a fried food is primarily due to the formation of pores by evaporation of water from the food surface [42]. The uptake of absorbed oil in food can range from 4 to 14% of the total weight, depending upon the food and type of frying medium, as reported by Andrikopoulos et al. [43] and the results are in conformity with this observation. A number of factors, like the time in and temperature of the oil, type of oil, and the shape and surface of the food and coatings, affect the oil uptake in fried foods [44]. Surface starch gelatinization due to the added gelatinized finger millet paste might have formed a layer that protects the food from oil absorption, as was reported by Califano and Calvelo [45]. Low oil absorption has also been reported by Kim et al. [46] by increasing the addition of preharvest-dropped apple pomace in instant fried noodles. Oil uptake while frying the snack was observed to be less compared to other fried products. This may be due to the reason that since the snack has been formulated with raw materials which have already undergone a preliminary cooking procedure, the chosen frying temperatures were high, both for par-frying as well as finish-frying. Moyano and Pedreschi [47] and Rojas-Gonzalez et al. [48] have argued that lower frying temperatures result in longer frying times and higher oil uptake. The acceptability of the ready-to-cook snack is attributable also to the crispness of the crust that developed due to deep frying, which is a factor of quality and freshness [49]. The increase in hardness may be attributed to the increase in fiber content of the product as well as the decrease in oil content of the fried snack as the level of TPP supplementation increases.

The lower scores for appearance are probably due to the dark color of the crust, which in turn is due to the lycopene content and dark color of FMF. The texture scores also showed a decrease after 15% supplementation with TPP, which might be attributed to the increased fiber content as well as less oil absorption by the snack. The snacks with 10% TP supplementation showed maximum overall acceptability, due to having highest scores for appearance, taste, and texture; the taste parameter had decreased scoring for supplementation beyond 10% due to the increased sourness of the product because of the tomato pomace. Based on these results, the RTC-GF snack with 10% TPP supplementation (T3) had the highest overall acceptability, and was therefore taken up for further storage studies.

FTIR spectroscopy helps to identify the presence or absence of specific functional groups as well as to support the results of chemical analysis [50]. The selected RTC-GF snack (T3) was subjected to FTIR spectroscopy (Figure 5a) for composition determination. The presence of a dominant peak at 2925 cm^{-1} confirms the presence of carbohydrates, being due to aliphatic C-H stretches [51], while the presence of phenolic compounds is confirmed by dominant peaks at 1376.6 cm^{-1} and 1378.5 cm^{-1} which are due to the presence of CH_3 stretch. Similarly, work by Jebitta et al. [52] had a peak at 2931 cm^{-1},

which they reasoned was associated with asymmetric and symmetric stretching modes of alkane C–H. The peak at 1261.1 cm^{-1} and 1241.4 cm^{-1} denotes an asymmetrical C-O-C stretch asserting the presence of phenolic compounds. The absorption bands at 1013.5 cm^{-1} and 1011.3 cm^{-1} are due to C-C and C-O stretching in addition C-O-H bending which are attributed to the structural changes in starch [53]. These bands arise mainly from carbohydrates of cellulosic origin [54]. The peak at 1745.2 cm^{-1} is due to C-O (esters) which indicates lipid characteristics [52]. The absorption bands in the range of 3290 cm^{-1} to 3373 cm^{-1} indicate the presence of hydroxyl groups and denote the OH bond stretch [55] which shows a decrease in percent transmittance in the finished product because of a loss of moisture in the sample product due to the extended frying time and high temperature. Similar data were reported by Gowthamraj et al. [56] and Gull et al. [57]. The presence of an absorption band at 1376–1378 cm^{-1} (C-CH_3 stretching) due to the CH bending vibration indicates the presence of cellulose and hemicellulose chemical structures which are the established components of tomato pomace [58]. Some stretching vibrations at 2854 cm^{-1} and 1745 cm^{-1} might be due to presence of non-starch constituents such as protein and fat [59]. The presence of phenolic compound peaks in the finished product confirms the positive contribution towards antioxidant potential of the final product. Most of the functional groups are retained in the RTC-GF (T3) product, as confirmed from the FTIR spectra of the finished product.

The complete nutritional profile of the RTC-GF snack (T3) and the control snack (T1) is enlisted under Table 3. Most of the vegetarian ready-to-cook frozen snacks available in the market have a protein content in the range of 2 to 3% whereas the RTC-GF snack (T3) has a protein content which is more than double this (6.9%) along with an adequate amount of fiber. As opposed to T1 having no TPP, the RTC-GF snack (T3) delivers a substantial amount of lycopene, which has 10 times the singlet-oxygen-quenching ability as that of α-tocopherol [60]. The processing temperature enhances the absorption of lycopene by altering the trans isomers to cis form [61], which is further enhanced by the addition of oil in the product, since lycopene is fat soluble. The enrichment with TPP in T3 resulted in a higher TPC and TFC of the snack as also reflected in significantly higher antioxidant activity (as DPPH, FRAP, ABTS and MCA). Similar results are reported by Isik and Topkaya, who also observed increased TPC and antioxidant activity in supplemented crackers in their work on tomato pomace supplementation [62]. Dewanto et al. [63] inferred from their studies that thermal processing enhanced the nutritional value of tomatoes and produced no significant changes in the TPC and TFC of tomatoes which helps in enhancing the antioxidant potential as well. The T3 sample also delivers good amounts of minerals, especially calcium, phosphorus, and potassium. Positive calcium content maintains healthy bones [64] while potassium prevents the onset of diabetes, renal, and cardiovascular diseases [65]. 'Western' diets have $\omega 6/\omega 3$ fatty acids in a ratio of up to 20:1, whereas this ratio should be low as this helps in the management of obesity [66]. The RTC-GF snack (T3) has a ratio of 7.1:1, which is quite reasonable and low, as a high ratio of $\omega 6/\omega 3$ is known to be a risk factor in cancers and coronary heart disease [67]. The essential amino acid composition profile of T3 shows that this RTC product is a source of good quality protein as it has valine, lysine, methionine, threonine, and tryptophan in good amounts, as recommended by the WHO [68]. Overall, it also provides sufficient energy (248.2 kcals) and hence good satiation level; thus, the RTC-GF snack (T3), besides being high on energy, also provides good nutritional value.

Lipid oxidation is often the determining factor during shelf studies of foods, since it is the cause of adverse changes in flavor and nutritive value, and has health implications as well. The peroxide value as well as the free fatty acid values of the RTC-GF snack (T3) show a very slight increase during the storage period but the values are well within the acceptable range for both the packaging materials (Figure 6a). Free fatty acids are the result of hydrolytic rather than oxidative rancidity and are used as an indicator of the storage stability of fried foods. A peroxide value of 2.0 meqO_2/kg of fat is normally considered to be low and fats are free of oxidative flavor at this point [69].

The sensory evaluation of the RTC-GF snack during the storage period shows little decrease in the overall acceptability scores of the product. During par frying, the amylose and amylopectin present in the raw material form a gel by losing their crystalline structure, which when cooled at low temperature undergoes retrogradation [70]. During the finish frying of the ready-to-cook frozen snack the retrogradation is reversed and the snack regains its original crispy nature, and this is corroborated by the good overall acceptability scores obtained by the snack which show a non-significant decrease in the values (Figure 6b). The overall acceptability of the product stored in HDPE pouches had higher scores. Hence, it is assumed that the product will show good acceptance beyond the storage study period as well, but further studies are needed to corroborate this.

5. Conclusions

Ready-to-cook frozen snacks are very popular in the market but have limited availability and choice under the gluten-free range. The developed RTC-GF product shows a very high index of acceptability with good storage stability and a very rounded nutritional profile that can very well cater to all sections of society, but specifically to gluten-sensitive people. Besides this, it is made by utilizing a byproduct of the processing industry and hence can help in reducing environmental pollution and attaining sustainability. The product with TPP at 10% level helped in lowering the oil uptake and enhancing the fiber, lycopene content, mineral, and antioxidant activity. Therefore, the utilization of tomato pomace powder in the development of products should be taken up for delivering foods with enhanced nutritional content. Further studies can be undertaken on making suitable interventions to reduce the acidity of the tomato pomace and thus enable its increased addition in food products without adversely affecting the sensorial characteristics.

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The Phenolic Composition of Hops (*Humulus lupulus* L.) Was Highly Influenced by Cultivar and Year and Little by Soil Liming or Foliar Spray Rich in Nutrients or Algae

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Abstract: The interest in expanding the production of hops outside the traditional cultivation regions, mainly motivated by the growth of the craft brewery business, justifies the intensification of studies into its adaptation to local growing conditions. In this study, four field trials were undertaken on a twenty-year-old hop garden, over periods of up to three years to assess the effect of important agro-environmental variation factors on hop phenol and phenolic composition and to establish its relationship with the elemental composition of hop cones. All the field trials were arranged as factorial designs exploring the combined effect of: (1) plots of different vigour plants × year; (2) plots of different plant vigor × algae- and nutrient-rich foliar sprays × year; (3) plot × liming × year; and (4) cultivars (Nugget, Cascade, Columbus) × year. Total phenols in hops, were significantly influenced by most of the experimental factors. Foliar spraying and liming were the factors that least influenced the measured variables. The year had the greatest effect on the accumulation of total phenols in hop cones in the different trials and may have contributed to interactions that often occurred between the factors under study. The year average for total phenol concentrations in hop cones ranged from 11.9 mg g^{-1} to 21.2 mg g^{-1}. Significant differences in quantity and composition of phenolic compounds in hop cones were also found between cultivars. The phenolic compounds identified were mainly flavonols (quercetin and kaempferol glycosides) and phenolic carboxylic acids (*p*-coumaric and caffeic acids).

Keywords: cultivars; foliar sprays; *Humulus lupulus*; liming; phenolic compounds; plant vigour

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1. Introduction

The most important hop (*Humulus lupulus* L.) compounds for brewing are resins and essential oils, which are responsible for beer bitterness and flavour. Both are synthesized in the lupulin glands of female cones [1,2]. Hop cones also contain other important compounds, such as polyphenols, which contribute to beer flavour, colour, taste and haze formation and have a strong antioxidant power [3,4]. Hop polyphenols include flavonols (e.g., quercertin and kaempferol), flavan-3-ol (e.g., catechins and epicatechins), phenolic acids (e.g., ferulic acid), prenylflavonoids (xanthohumol, isoxanthohumol, desmethylxanthohumol, 6- and 8-prenylnaringenin), multifidus glycosides and resveratrol [1,5,6]. The polyphenolic fraction of hops is so complex that researchers still continue to identify compounds [1,7].

In Portugal, hop plants occur spontaneously along riverbanks, in particular in the north of the country, a region that has been found to have some ecological potential for hop production [8]. The crop was introduced into the country in the early 1960s and currently the national production is located mainly in the Bragança district in the northeast of the country. Nugget, a bitter cultivar, is produced on all the local farms, being destined for a national brewing company. However, local farmers are currently interested in growing aroma cultivars due to the recent growth of the craft beer industry both at home and abroad. According to the Euromonitor [9] report "Beer in Portugal", dozens of craft brewers launched different craft beer products in 2019. Currently Cascade and Columbus are some of the aroma cultivars that regional farmers are starting to experiment with. With the expansion of the craft beer market, new opportunities arise for small-scale growers, producing and supplying desirable cultivars at more favourable prices [10]. The growing of aroma cultivars for the craft beer market is probably also more suited to the Portuguese production structure, which is based on small-sized plots. To reduce the risk of failure, as occurred during recent years with the bitter cultivars grown for the conventional beer industry, greater knowledge should be applied to the Portuguese production system. In other Mediterranean countries, hops also attracted the attention of producers and researchers, and recent work has shown good suitability of different cultivars both for the beer industry [11] and for the production of fresh edible shoots [12–14]. Thus, an important step for Portuguese farmers is comparing the agronomic performance of other cultivars sought by the craft beer industry with the well-established Nugget. The response of hops to soil pH, for instance, is also important to be understood since pH is an important factor in hop production [15,16]. Farmers are currently starting to use foliar sprays to complement their fertilization programmes, given the potential beneficial effects of such products in crop production and quality [17,18]. There is also growing interest in the use of biofertilizer formulations from readily available materials to improve soil conditions and plant yield [19,20]. Over the years, some fields have been showing patches of poorly developed plants that reduce overall productivity and farmers' incomes [21]. Strictly speaking, the cause of these underdeveloped plant is not yet clearly known. There are no signs of phytosanitary problems. Thus, it is important to look at these patches of poorly developed plants and observe the effect of foliar sprays on hop cone quality.

As mentioned above, previous studies have shown that hops can be a promising crop for Mediterranean environments, although it is necessary to improve several aspects of the cropping technique [11]. Thus, this study aims to carry out a set of experimental trials to test important factors (plant vigour, foliar sprays, liming, cultivar and year) that can influence the quality of the cones and particularly phenol concentration and phenolic composition. A high content of phenols is a positive trait of hop cones, due to their bioactive effect, which contributes to beer quality [4–6]. The field trials included four factorial designs exploring the combined effects of (1) plots of different vigour plants and year, (2) plots of different vigour plants, algae- and nutrient-rich foliar sprays and year, (3) plots, liming and year and (4) cultivars and year. From these trials, the concentration of polyphenols in hop cones is reported. The samples presenting the higher polyphenolic content from each of the trials, were selected for phenolic characterization. Furthermore, the relationship between total phenols and nutrient concentration in hop cones was evaluated through a principal component analysis (PCA) and correlation analysis. The results of the elemental composition of hop cones, already reported in previous studies [22–24], were used to evaluate their relationship with hop phenols. Data on dry matter (DM) yield and hop acids from these experiments have also been reported [21–24], but the relevant information to understand the accumulation of phenols in plants in response to the different factors of variation was discussed here. In short, the ultimate objective of this research was to obtain useful data for both hop producers and the craft beer industry.

2. Materials and Methods

2.1. Experimental Conditions

The field trials were conducted on hop farms located in Bragança (41°41′33.6″ N, 6°44′32.7″ W, and 850 m above sea level), north-eastern Portugal, from 2016 to 2018. The region benefits from a Mediterranean-type climate, with an average annual temperature and precipitation of 12.7 °C and 772.8 mm, respectively [25]. Data on average monthly temperatures and precipitation during the experimental period are shown in Figure 1.

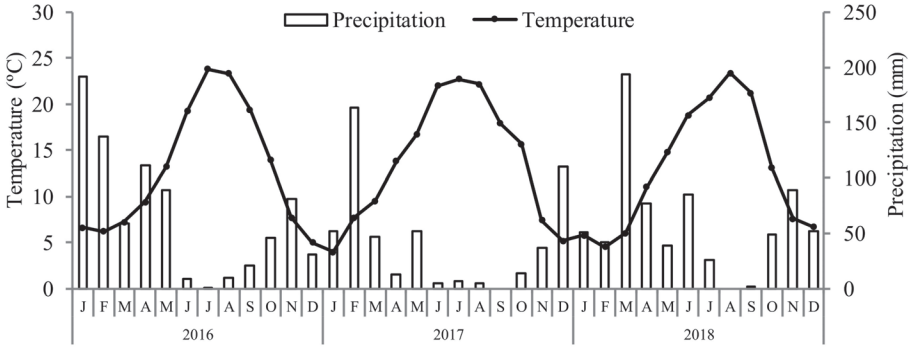

Figure 1. Average monthly temperature and precipitation during the three years of the study.

Six plots, named here as Plot 1 (~0.5 ha), Plot 2 (~0.5 ha), Plot 3 (~4 ha), Plot 4 (~2 ha), Plot 5 (~2 ha) and Plot 6 (~2 ha), were used in this experimental protocol. The classification of the fields was made with the farmers' help and was based on the crop growth and yield in the previous years. In Plot 1, the plants were classified as weak vigour plants, as the hop bines did not reach 4 m in height. In Plot 2, the growth of the plants was classified as fair, as the plants did not reach the top of the pole (7 m). In Plot 3, the vigour of the plants was classified as good, as the hop bines exceeded 7 m in height, but the volume of the canopies, aboveground biomass and cone production were clearly below optimal. In the Plots 4, 5 and 6, the vigour of plants was classified as very good vigour, since the hop bines reached a full size and produced abundantly.

Before the installation of the trials, all the plots were analysed for soil properties. The soil samples were collected in three replicates at 0–0.20 m depth. Each replicate was a composite sample, prepared from soil collected from 15 random points. The samples were oven-dried at 40 °C and sieved in a mesh of 2 mm. Thereafter, they were analysed for pH_{H2O} (soil: solution, 1:2.5), cation-exchange capacity (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley–Black method), extractable P and K (Egner–Riehm method) and soil separates [26]. The results of the soil analysis are presented in Table 1.

Table 1. Selected soil properties (average ± standard deviation; n = 3) determined just before the start of the experiments from soil samples collected in plots of different plant vigour (weak, fair, good and very good) at 0–0.20 m depth.

Soil Properties	Plot 1 (Weak)	Plot 2 (Fair)	Plot 3 (Good)	Plot 4 (Very Good)	Plot 5 (Very Good)	Plot 6 (Very Good)
Clay (%) [a]	27.0 ± 5.8	35.0 ± 4.6	22.1 ± 2.0	18.1 ± 1.8	17.7 ± 2.1	16.8 ± 0.9
Silt (%) [a]	21.6 ± 10.7	22.8 ± 4.4	5.1 ± 1.6	35.5 ± 5.7	24.7 ± 3.2	24.3 ± 3.1
Sand (%) [a]	51.4 ± 16.5	42.2 ± 2.4	72.8 ± 18.4	46.4 ± 6.9	57.6 ± 4.8	58.9 ± 8.5
pH_{H2O} [b]	5.8 ± 0.12	5.8 ± 0.04	5.5 ± 0.10	5.1 ± 0.13	5.8 ± 0.03	5.3 ± 0.03
Organic carbon (g kg^{-1}) [c]	13.4 ± 0.20	15.7 ± 0.10	7.6 ± 0.04	14.5 ± 0.20	17.2 ± 0.08	19.4 ± 0.07
Extract. P (mg P_2O_5 kg^{-1}) [d]	283 ± 45	452 ± 34	191 ± 28	213 ± 28	296 ± 20	289 ± 16
Extract. K (mg K_2O kg^{-1}) [d]	116 ± 7	193 ± 9	111 ± 6	286 ± 5	332 ± 9	162 ± 6
Exch. Ca (cmolc kg^{-1}) [e]	14.8 ± 1.84	23.3 ± 1.39	10.7 ± 0.17	2.7 ± 0.46	4.9 ± 0.24	4.6 ± 0.18
Exch. Mg (cmolc kg^{-1}) [e]	4.8 ± 0.84	9.5 ± 1.22	2.7 ± 0.07	0.5 ± 0.04	0.7 ± 0.03	0.6 ± 0.02
Exch. K ($cmol_c$ kg^{-1}) [e]	0.3 ± 0.02	0.5 ± 0.04	0.2 ± 0.01	0.5 ± 0.08	0.6 ± 0.03	0.3 ± 0.01
Exch. Na ($cmol_c$ kg^{-1}) [e]	0.2 ± 0.05	0.6 ± 0.05	0.1 ± 0.01	0.3 ± 0.06	0.06 ± 0.01	0.05 ± 0.01
Exch. acidity ($cmol_c$ kg^{-1}) [e]	0.3 ± 0.03	0.3 ± 0.02	0.2 ± 0.03	0.6 ± 0.13	0.2 ± 0.02	0.4 ± 0.03
CEC ($cmol_c$ kg^{-1}) [e]	20.7 ± 2.64	34.4 ± 2.56	14.0 ± 0.21	5.1 ± 0.37	6.7 ± 0.28	6.5 ± 0.17

[a] Pipette method; [b] Potentiometry; [c] Walkley–Black; [d] Egner–Riehm; [e] Ammonium acetate, pH 7.

All the plots where the experiments took place were grown on a high trellis system supported by concrete poles and a network of steel cables placed at a height of 7 m. The hop bines were guided from the ground to the upper net with nylon threads. At planting, the seedlings were spaced at 2.8 m × 1.6 m between and within rows. Two tutors emerged from each original place where the seedlings were planted, giving rise to a density of 2232 plants per hectare, which were trained in Spring into two twin canopies.

The plots were irrigated by flooding the space between rows. Farmers estimate the average use of 6000 m^3 of water per hectare and per year, equivalent to 600 mm. From the end of May to mid-August they perform an average of 10 watering events of 60 mm each.

The floor was managed by tillage (3 to 4 passes per year), which has a double function of controlling weeds and removing the superficial crust caused by this irrigation method allowing a better water infiltration at subsequent irrigation events.

All the plots received an annual fertilization plan consisting of the application of a compound NPK (7:14:14) fertilizer late in winter (just before plant regrowth from winter resting period) at a rate of ~500 kg ha^{-1}. Thereafter, during the growing season, two side dress N applications were performed by using ~200 kg ha^{-1} of ammonium nitrate (27% N) (applied when plants were close to reach the top wire) followed by ~450 kg ha^{-1} of calcium nitrate (15.5% N) (applied at early flowering).

2.2. Experimental Designs

The experimental design was divided into four field trials arranged as factorial designs with six replications (six twin canopies of three plants). The plants were randomly selected in the corresponding experimental plots when they reached 3 m in height in the plots of higher vigour plants.

Experiment 1 consisted of a factorial design (two factors) including plots of plants of different vigour (weak, fair, good and very good) and years (2016, 2017 and 2018). The classification of the vigour of the plants was made with the farmers help as above mentioned. The plots were planted with the Nugget cultivar and were installed ~20 years ago.

Experiment 2 consisted of a factorial design (three factors) of plots of different plant vigour (weak, fair, good and very good), foliar sprays (algae- and nutrient-rich foliar sprays and control) and years (2017 and 2018).

The algae-rich foliar spray (Algae) is a solution containing 15% (w/w) the algae *Ascophyllum nodosum* (L.) Le Jolis, applied at a rate of 2 L ha^{-1} (diluted in 1500 L of water) three times during the growing season, at the phenological stages of inflorescence

emergence, flowering, and beginning of the development of cones (on 20 June, 10 July and 27 July 2017, and 20 June, 8 July and 24 July 2018, respectively). In spite of the differences on plant vigour of the different plots, the phenological stage of the plants was similar.

The nutrient-rich foliar spray (Fnut) is a mixture of *A. nodosum* (1.4% *w*/*w*) enriched with macro- and micronutrients containing (*w*/*w*) 12% N, 6% P_2O_5, 4% K_2O, 0.025% B, 0.1% Fe-EDTA, 0.05% Cu-EDTA, 0.05% Zn-EDTA, and 0.05% Mn-EDTA. This fertilizer was applied at a rate of 3.5 L ha^{-1} (diluted in 1500 L of water) on the dates reported for Algae. In each plot the foliar sprays were applied in four rows and the six twin canopies of each treatment were sampled in the two interior rows. The plots where this experiment was carried out were the same reported for experiment 1, although in a different part of the plots.

Experiment 3 was arranged as a factorial design (three factors) and included hop plots (two) of good vigour plants (Plots 5 and 6), liming (limed and not limed) and years (2017 and 2018). The limestone (55% $CaCO_3$, 28% CaO and 20% MgO) was applied at a rate of 1000 kg ha^{-1} in February 2017 and incorporated into the soil with a cultivator. Both fields, ~2 ha each, are of the Nugget cultivar and they are ~20 years old. As the study was carried out as on-farm research, liming was carried out in a larger part of the area, with only four rows of ~150 m remaining for the control treatment, and the plants were sampled from the internal rows of the treated or untreated plots.

Experiment 4 was a factorial of two factors: cultivars (Nugget, Cascade and Columbus) and year (2017 and 2018). This experiment was carried out in Plot 4, in which part of the plot was installed with several different cultivars, each one occupying a row of ~150 m. This hop field was planted in 2014. An overview of the experimental design is shown in

2.3. Plant Sampling and Tissue Analysis for Elemental Composition

Plant material was collected at harvest and subsamples of fresh cones were carried to the laboratory, oven-dried at 70 °C and thereafter ground for laboratory analysis. Tissue analysis for elemental composition was performed by Kjeldahl (N), colorimetry (P and B), flame emission spectroscopy (K), and atomic absorption spectroscopy (Ca, Mg, Cu, Fe, Zn, and Mn) methods [27], after the samples were digested with nitric acid in a microwave (MARSXpress CEM).

2.4. Analysis of Total Phenolics

Hop cone samples were ground in a Cyclotec mill, with a 1 mm mesh screen, to obtain a fine powdered sample. Infusion preparation was performed by using 1 g of fine powdered hop sample, which was added to 100 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered. Total phenols were determined in a total of 204 samples (36 samples from Experiment 1, 72 samples from Experiment 2, 72 samples from Experiment 3 and 24 samples from Experiment 4). The extracts obtained were diluted 1:1. Folin–Ciocalteu's assay, briefly, 0.5 mL of each diluted extract was mixed with the Folin–Ciocalteu reagent (2.5 mL). After 3 min, they were saturated with sodium carbonate solution (2 mL) and the reaction was kept in a water bath at 40 °C for 30 min. The absorbance was read at 765 nm (PG Instruments T80 UV/VIS Spectrophotometer, QLabo, Portugal). Gallic acid was used to prepare the standard curve and the results were expressed as mg of gallic acid equivalents (GAEs) per g of dry matter of hop cones. The analysis of total phenols in each sample was carried out in triplicate.

2.5. HPLC Analysis

The samples of phenolic extracts with the highest content of total phenols from each trial and 2017 (experiment 1 also from 2016) were selected and analysed for their phenolic compound content in the directly infused extracts, and then filtered using 0.22 μm disposable disk filters. Phenolic compounds were determined in a total of 36 samples from all the experiments. The operating conditions were followed according to that previously described by Bessada et al. [28] using a HPLC system (Dionex Ultimate 3000 UPLC, Thermo

Scientific, San Jose, CA, USA) coupled with a diode-array detector (DAD, using 280 and 370 nm as preferred wavelengths) and a Linear Ion Trap (LTQ XL) mass spectrometer (MS, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The separation was made in a Waters Spherisorb S3 ODS-2 C18 column (3 µm, 4.6 mm × 150 mm; Waters, Milford, MA, USA). Tentative phenolic compound identification was made according to their UV and mass spectra and retention times compared with commercial standards when available or using reported data from the literature. For the quantitative analysis of phenolic compounds, a 7-level calibration curve was obtained by injecting known concentrations. The results were expressed in mg per kg of fresh weight (fw), as mean ± standard deviation of three independent analyses. Figure 2.

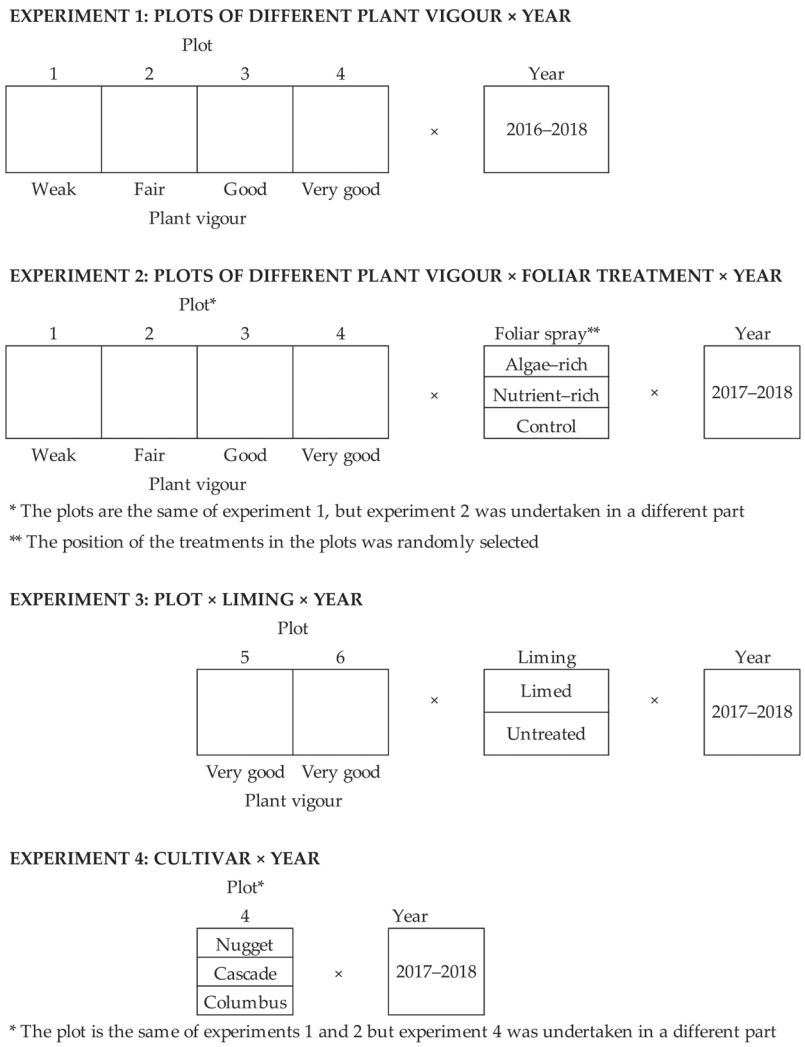

Figure 2. Schematic view of the experiments, including the four field trials reported in this study.

2.6. Statistical Analysis

Data were firstly tested for normality and homogeneity of variance using Shapiro–Wilk and Bartlett's tests, respectively. Thereafter, data were subjected to two- or three-way ANOVA according to the experimental design using SPSS v. 25.0 programme. When

the means differed significantly, they were separated by Student's or Tukey HSD tests ($\alpha = 0.05$), when the factors were applied at two or more levels, respectively. A PCA was performed with the object principal normalization method on data collected from 2016 to 2018 regarding total phenols and nutrient concentration in cones. The principal components were retained considering the eigenvalues superior to 1 and the scree plot. Internal consistency was measured with Cronbach's alpha. In addition, the scores of each one of the PCA components were calculated as a function of plant vigour, foliar treatment, limestone treatment, cultivars and year, and subjected to analysis of variance, using the Tukey–Kramer HSD test ($\alpha = 0.05$) to compare averages for each trial and year. A correlation analysis was applied to the same data as the PCA analysis with the Spearman coefficient.

3. Results

3.1. Total Phenols in Cones

In the factorial experiment of plant vigour × year, a significant interaction was found for total phenols (Figure 3), meaning that the response of this variable to the field of plants of different vigour depended on the year and/or vice versa. Observing the effect of each factor separately, significant differences were found between years but not between fields. In 2017 total phenols were particularly higher than in 2016 and 2018. The average values were 19.0, 11.9 and 15.1 mg g^{-1} in 2017, 2018 and 2016, respectively.

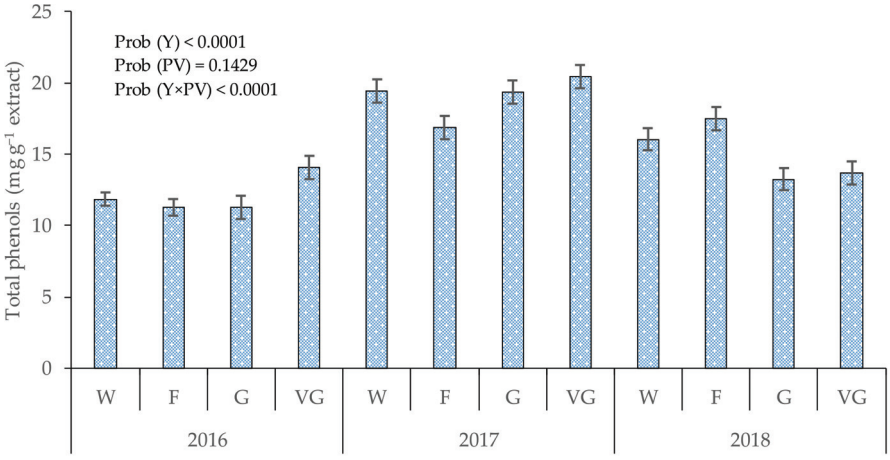

Figure 3. Total phenols as a function of year and hop plant vigour (weak—W, fair—F, good—G and very good—VG) and year. Error bars are the standard errors ($\alpha = 0.05$).

In the factorial experiment of plant vigour × foliar treatment × year, a significant interaction was found for total phenols for the combination of the three factors and for plant vigour × year and foliar treatment × year (Figure 4). Thus, the year seems to be the factor that adds more variability to the results, influencing the accumulation of total phenols in plants of different vigor and subject to different foliar treatment. By analysing the factors separately, differences in total phenols between plots were found, but without any relation to the vigour of the plants. Foliar sprays did not cause a significant effect on total phenols, but in 2017 the values were significantly higher than those of 2018. The average values were 21.2 and 14.7 mg g^{-1}, respectively, in 2017 and 2018.

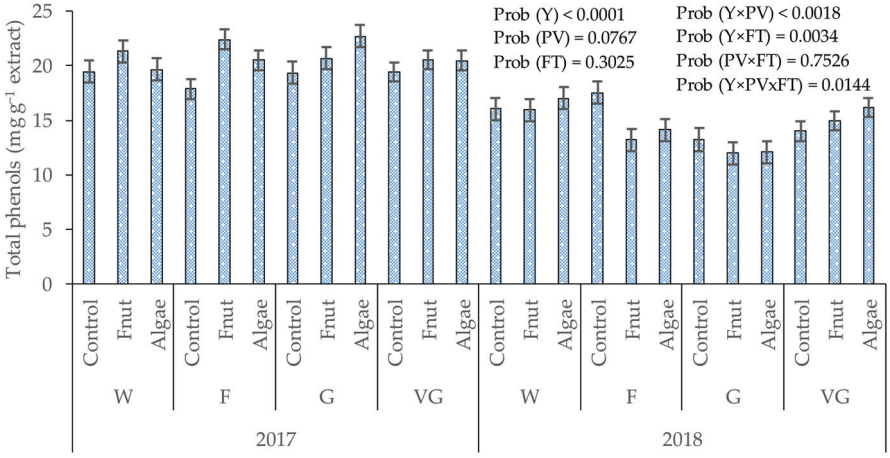

Figure 4. Total phenols as a function of year, plant vigour (weak—W, fair—F, good—G and very good—VG) and foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control). Error bars are the standard errors ($\alpha = 0.05$).

In the factorial experiment of different plots × liming × year, significant interaction for total phenols only occurred between plot × year (Figure 5), meaning that total phenol accumulation in plants from different plots was dependent on the year effect. In this experiment, the effect of the plot and year was not statistically significant, and lime's application significantly reduced the content of total phenols. The average values of total phenols were 17.8 and 16.5 mg g^{-1}, respectively, in control and limed plots.

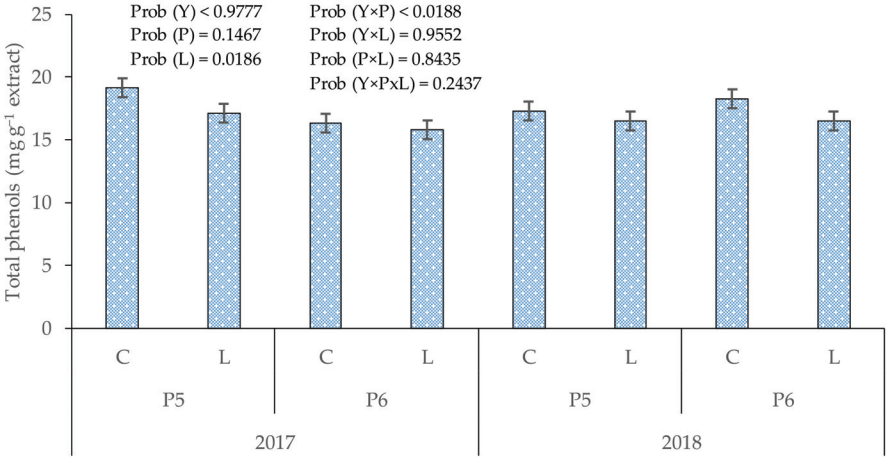

Figure 5. Total phenols as a function of year, plot (P5 and P6) and liming (L, limed; and C, not limed). Error bars are the standard errors ($\alpha = 0.05$).

In the factorial experiment cultivars × year, significant interaction was found for total phenols, which means that the response of the cultivars depended on the year (Figure 6). A separate observation of the effect of each of the factors indicated that Nugget showed significantly lower values than Columbus and Cascade, and the values of 2017 were significantly higher than those of 2018. The average values of Nugget, Columbus and Cascade were 16.7, 19.9 and 19.6 mg g^{-1}, respectively, and the average values of 2017 and 2018 were 19.9 and 17.6 mg g^{-1}, respectively.

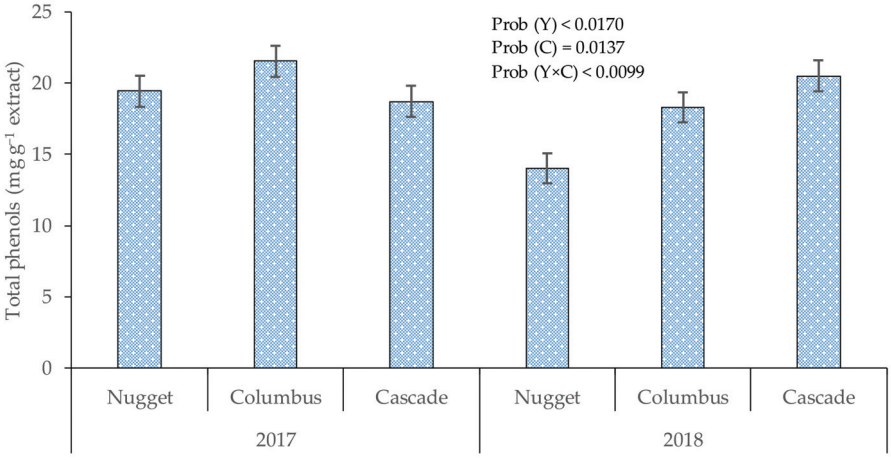

Figure 6. Total phenols as a function of year and cultivar. Error bars are the standard errors (α = 0.05).

3.2. Principal Component Analysis

The PCA applied to data collected from 2016 to 2018 concerning total phenols and nutrient concentration in hop cones resulted in four principal components (PC1 to PC4), which accounted for 70.02% of the variance explained. The main differences in the variance explained were between PC1 (23.35%) and PC4 (11.77%). All variables presented high scores for at least one, or more than one, PC (Table 2). The positive association with N, P, Mg and negative association with K seems to explain greater variance. The higher loading of total phenols was negative and registered in PC3 (−1.606), but scores were also high in PC4 (0.753) and in PC1 (−0.730). These results seem to indicate a negative association of total phenols with Zn and B.

Table 2. PCA results for total phenols and nutrient concentrations on hop cones from 2016 to 2018.

	PC1	PC2	PC3	PC4
Eigenvalue	2.569	2.028	1.811	1.294
Cronbach's Alpha	0.672	0.558	0.492	0.250
Explained variance	23.35	18.44	16.46	11.77
Cumulative variance	23.35	41.79	58.25	70.02
Variable Loadings				
Total phenols	**−0.730**	−0.359	**−1.606**	**0.753**
Nitrogen	**1.391**	**−1.195**	0.369	0.618
Phosphorus	**1.516**	−0.091	−0.555	−0.074
Potassium	**−1.206**	−0.867	0.216	**1.134**
Calcium	0.272	**−1.982**	−0.014	0.311
Magnesium	**1.319**	−0.363	−0.584	**1.707**
Iron	0.054	**1.226**	0.822	**1.147**
Manganese	−0.868	−0.572	**1.136**	**1.238**
Copper	0.361	**1.265**	**1.071**	**1.363**
Zinc	**1.277**	−0.179	**1.409**	−0.823
Boron	−0.668	**−1.077**	**1.578**	−0.605

PC—principal component; values in bold correspond to the higher loadings of each variable in the respective PC.

Correlation analysis (Table 3) indicates total phenols significantly and negatively correlated with Zn followed by Cu, N and Fe in decreasing order. Positive and significant correlations of total phenols with other nutrients were not recorded. On the other hand, cone N concentration presented positive correlations with other nutrients and most significantly with Mg and P, whereas K was significant and positively correlated with Mn.

Table 3. Correlation matrix of total phenols (TPH) and nutrient in hop cones, with Spearman correlation coefficients.

	TPH	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
TPH	1										
N	−0.223 **	1									
P	−0.008	0.412 **	1								
K	0.114	−0.052	−0.266 **	1							
Ca	0.138	0.393 **	0.103	0.078	1						
Mg	−0.051	0.513 **	0.295 **	0.032	0.147	1					
Fe	−0.186 *	−0.077	−0.069	−0.126	−0.220 **	0.232 **	1				
Mn	0.074	−0.016	−0.298 **	0.402 **	0.019	−0.191 *	0.096	1			
Cu	−0.295 **	0.028	0.047	−0.139	−0.356 **	0.294 **	0.520 **	0.116	1		
Zn	−0.456 **	0.345 **	0.371 **	−0.269 **	0.138	0.005	0.100	−0.071	0.197 *	1	
B	−0.105	0.074	−0.176 *	0.250 **	0.348 **	−0.273 **	−0.058	0.311 **	−0.070	0.223 **	1

*, ** Significant correlations according to selected significance levels, 0.05 and 0.01, respectively.

3.3. Phenolic Compounds Identification and Quantification

Data on the chromatographic characteristics (retention time, UV in the maximum absorption, molecular ion, and main MS^2 fragments) and tentative identification of the phenolic compounds found in the extracts of hop cones are described in Table 4. A total of 13 phenolic compounds were tentatively identified in the samples, namely, 5 phenolic acids (*p*-coumaroyl- and caffeoylquinic acid derivatives) and 8 *O*-glycosylated flavonoids (quercetin and kaempferol derivatives).

Table 4. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectral data, and identification of the phenolic compounds present in hop cones extract: 3-CQA (3-*O*-Caffeoylquinic acid), *cis* 3-*p*-CoQA (*cis* 3-*p*-Coumaroylquinic acid), *trans* 3-*p*-CoAD (*trans* 3-*p*-Coumaroylquinic acid), 4-CQA (4-*O*-Caffeoylquinic acid), 5-CQA (5-*O*-Caffeoylquinic acid), Q-3-2Rh-Ru (Quercetin-3-*O*-(2-rhamnosyl)-rutinoside), K-3-2Rh-Ru (Kaempferol-3-*O*-(2-rhamnosyl)-rutinoside), Q-3-Ru (Quercetin-3-*O*-rutinoside), Q-3-H (Quercetin-3-*O*-hexoside), Q-3-6M-G (Quercetin-3-*O*-(6-O-malonyl)-glucoside), K-3-Ru (Kaempferol-3-*O*-rutinoside), K-3-G (Kaempferol-3-*O*-glucoside), K-3-6M-G (Kaempferol-3-*O*-(6-O-malonyl)-glucoside).

Peak	Tentative Identification	R_t (min)	λ_{max} (nm)	[M-H] (*m*/*z*)	MS^2
1	3-CQA	4.80	340	353	191(100), 179(47), 173(3), 135(7)
2	*cis* 3-*p*-CoQA	5.46	310	337	191(10), 163(100), 119(10)
3	*trans* 3-*p*-CoAD	6.31	310	337	191(53), 163(100), 119(12)
4	4-CQA	6.86	325	353	191(14), 179(53), 173(100), 135(2)
5	5-CQA	7.25	323	353	191(100), 179(15),173(5), 135(2)
6	Q-3-2Rh-Ru	14.6	330	755	609(45), 591(94), 573(12), 489(70), 301(100)
7	K-3-2Rh-Ru	16.59	330	739	593(26), 575(100), 393(8), 285(38)
8	Q-3-Ru	17.86	353	609	301(100)
9	Q-3-H	19.06	351	463	301(100)
10	Q-3-6M-G	20.29	353	549	505(100), 463(25), 301(50)
11	K-3-Ru	21.15	347	593	285(100)
12	K-3-G	22.52	345	447	285(100)
13	K-3-6M-G	24.72	347	533	489(100), 285(20)

Data on the quantification of phenolic compounds in the three different cultivars of hop cones are described in Tables 5 and 6. An example phenolic profile chromatogram of the Cascade cultivar is presented in Figure 7. The quantification of the individual phenolic compounds from the first trial (Plant Vigour × Year) revealed that some of the compounds were not detected in plants with good and very good vigour, in particular, *O*-glycosylated kaempferol derivatives and caffeoylquinic acid derivatives (data not shown). Plants of weak vigour were generally higher in quercetin and kaempferol derivatives. The concentration of phenolic compounds in hop cones was very similar among foliar fertilizer treatments,

although for most of the compounds the values were slightly higher in the control treatment and slightly lower in the algae treatment (Table 5). In comparison with the control, plants on limed soil presented a significantly higher concentration of kaempferol-3-*O*-(2-rhamnosyl)-rutinoside and 4-*O*-caffeoylquinic acid though not significantly.

Table 5. Phenolic compound quantification (mean ± standard deviation) in hop cone samples from 2017 as a function of foliar treatments (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control) and liming: 3-CQA (3-*O*-Caffeoylquinic acid), *cis* 3-*p*-CoQA (*cis* 3-*p*-Coumaroylquinic acid), *trans* 3-*p*-CoAD (*trans* 3-*p*-Coumaroylquinic acid), 4-CQA (4-*O*-Caffeoylquinic acid), 5-CQA (5-*O*-Caffeoylquinic acid), Q-3-2Rh-Ru (Quercetin-3-*O*-(2-rhamnosyl)-rutinoside), K-3-2Rh-Ru (Kaempferol-3-*O*-(2-rhamnosyl)-rutinoside), Q-3-Ru (Quercetin-3-*O*-rutinoside), Q-3-H (Quercetin-3-*O*-hexoside), Q-3-6M-G (Quercetin-3-*O*-(6-O-malonyl)-glucoside), K-3-Ru (Kaempferol-3-*O*-rutinoside), K-3-G (Kaempferol-3-*O*-glucoside), K-3-6M-G (Kaempferol-3-*O*-(6-O-malonyl) glucoside).

Phenolic Compounds (mg kg^{-1}, dw)	**Foliar Treatment**			**Limestone Treatment**	
	Fnut	**Algae**	**Control**	**Limed**	**Control**
3-CQA	37.1 ± 16.4a	39.8 ± 15.7a	40.5 ± 10.4a	24.4 ± 7.0a	21.5 ± 6.4a
cis 3-*p*-CoQA	27.9 ± 3.0a	23.8 ± 6.9a	28.0 ± 3.9a	5.7 ± 3.6a	5.8 ± 7.1a
trans 3-*p*-CoAD	23.6 ± 6.9a	16.0 ± 6.0a	19.0 ± 4.1a	1.1 ± 2.2a	4.2 ± 7.3a
4-CQA	24.4 ± 1.7a	23.2 ± 1.0a	25.3 ± 1.9a	24.5 ± 4.8a	7.6 ± 15.2a
5-CQA	31.1 ± 7.8a	26.1 ± 3.5a	28.7 ± 3.7a	5.3 ± 10.7a	5.6 ± 11.2a
Q-3-2Rh-Ru	47.5 ± 0.2a	47.0 ± 0.3a	47.2 ± 0.2a	46.7 ± 0.4a	47.7 ± 1.4a
K-3-2Rh-Ru	46.5 ± 0.1a	46.5 ± 0.2a	46.5 ± 0.1a	46.5 ± 0.2a	11.6 ± 23.2b
Q-3-Ru	56.1 ± 0.8a	54.5 ± 4.0a	58.5 ± 1.5a	50.6 ± 1.1a	50.5 ± 2.1a
Q-3-H	72.6 ± 5.1a	64.6 ± 4.6a	71.5 ± 1.5a	54.6 ± 4.5a	56.6 ± 6.7a
Q-3-6M-G	93.1 ± 4.8a	84.9 ± 9.0a	96.5 ± 6.4a	70.2 ± 3.9a	66.1 ± 10.3a
K-3-Ru	50.2 ± 1.3a	49.6 ± 1.5a	51.0 ± 0.3a	47.5 ± 0.7a	46.9 ± 0.5a
K-3-G	52.1 ± 0.6a	50.8 ± 1.1a	52.3 ± 0.6a	48.0 ± 0.4a	36.2 ± 24.2a
K-3-6M-G	59.1 ± 3.5a	57.3 ± 3.3a	62.3 ± 2.7a	52.4 ± 1.2a	51.3 ± 3.1a
Total phenolic compounds	**621.4 ± 32.8a**	**584.1 ± 52.0a**	**627.2 ± 13.6a**	**477.6 ± 25.4a**	**414.0 ± 81.0a**

Means followed by the same letter are not statistically different by Tukey HSD (Foliar Treatment) or *t*-Student (Limestone treatment) tests (α = 0.05).

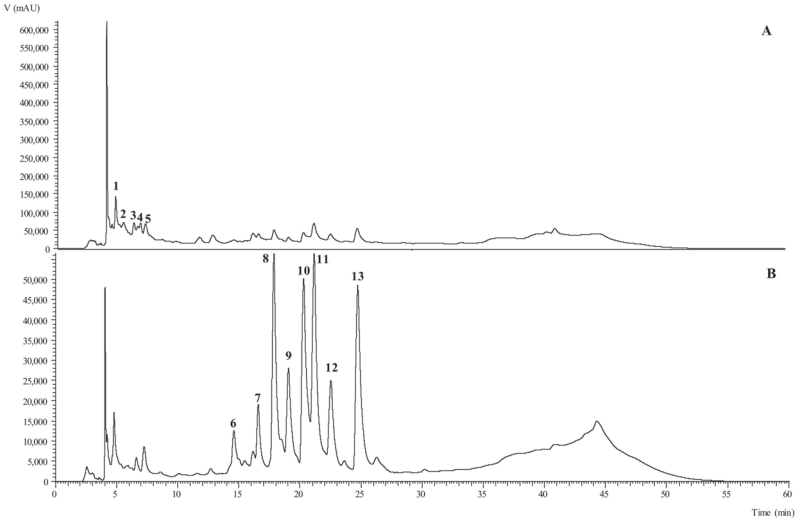

Figure 7. Chromatographic profile obtained at 280 nm (**A**) and 370 nm (**B**) of a hop cone extract (Cascade cultivar). The peaks 1 to 13 correspond to the phenolic compounds identified in Table 4.

Table 6. Phenolic compound quantification (mean ± standard deviation) in hop cone samples from 2017 as a function of the cultivar: 3-CQA (3-*O*-Caffeoylquinic acid), *cis* 3-*p*-CoQA (*cis* 3-*p*-Coumaroylquinic acid), *trans* 3-*p*-CoAD (*trans* 3-*p*-Coumaroylquinic acid), 4-CQA (4-*O*-Caffeoylquinic acid), 5-CQA (5-*O*-Caffeoylquinic acid), Q-3-2Rh-Ru (Quercetin-3-*O*-(2-rhamnosyl)-rutinoside), K-3-2Rh-Ru (Kaempferol-3-*O*-(2-rhamnosyl)-rutinoside), Q-3-Ru (Quercetin-3-*O*-rutinoside), Q-3-H (Quercetin-3-*O*-hexoside), Q-3-6M-G (Quercetin-3-*O*-(6-O-malonyl)-glucoside), K-3-Ru (Kaempferol-3-*O*-rutinoside), K-3-G (Kaempferol-3-*O*-glucoside), K-3-6M g (Kaempferol-3-*O*-(6-O-malonyl)-glucoside).

Phenolic Compounds (mg kg^{-1} Dry Matter)	**Cultivar**		
	Nugget	**Columbus**	**Cascade**
3-CQA	40.5 ± 10.4a	32.6 ± 5.9a	40.7 ± 9.9a
cis 3-*p*-CoQA	28.0 ± 3.9a	24.6 ± 14.9a	11.6 ± 6.0a
trans 3-*p*-CoAD	19.0 ± 4.1a	15.1 ± 4.9ab	9.0 ± 2.5b
4-CQA	25.3 ± 1.9ab	28.2 ± 7.9a	14.7 ± 3.2b
5-CQA	28.7 ± 3.7ab	22.1 ± 3.2b	32.1 ± 2.9a
Q-3-2Rh-Ru	47.2 ± 0.2b	46.8 ± 0.2b	50.3 ± 0.6a
K-3-2Rh-Ru	46.5 ± 0.1b	46.6 ± 0.1b	54.5 ± 2.4a
Q-3-Ru	58.5 ± 1.5b	52.3 ± 1.4b	72.4 ± 5.2a
Q-3-H	71.5 ± 1.5a	62.3 ± 4.9b	56.9 ± 1.6b
Q-3-6M-G	96.5 ± 6.4a	77.2 ± 11.4ab	71.5 ± 4.7b
K-3-Ru	51.0 ± 0.3b	51.5 ± 1.4b	74.2 ± 3.1a
K-3-G	52.3 ± 0.6b	54.9 ± 2.9ab	58.5 ± 2.2a
K-3-6M-G	62.3 ± 2.7a	71.3 ± 10.7a	76.1 ± 4.8a
Total phenolic compounds	**627.2 ± 13.6a**	**585.3 ± 61.8a**	**622.6 ± 35.7a**

Means followed by the same letter are not statistically different by Tukey HSD tests ($\alpha = 0.05$).

Between cultivars, the differences in phenolic compound quantification were significant for most of the compounds, though not for the total sum of phenolic compounds. Cascade presented lower concentrations of *p*-coumaroylquinic acid (*p*-CoQA) and 4-*O*-caffeoylquimic acid (4-CQA), but was generally higher in quercetin and kaempferol derivatives (Table 6). Nugget and Columbus were overall very similar in their phenolic profile.

4. Discussion

4.1. Total Phenols in Hop Cones

In the four factorial experiments, a significant interaction was found between two or three factors of each experiment for several traits related to total phenols in the cones. This means that the effect of a factor on a given variable was dependent on the other(s) factor(s) under study, and the year was the factor with greatest influence. The accumulation of total phenols in cones in plants of different vigour, in those subject to different foliar treatments and grown in different plots, and between different cultivars was dependent on the year. Abram et al. [29] also reported that the year influenced the phenolic content of hop cones of different cultivars and of hop plants grown in different locations (Slovenia, Austria, Czech Republic). The year effect results from the combination of important environmental variables, such as precipitation, temperature, solar radiation, etc., which are able to influence physiological and biochemical processes in plants and also the efficiency of foliar nutrition [30]. The year had a marked effect on total phenol content. Total phenols showed lower values in 2018 in most experiments in comparison with 2017.

During important phases of the growing season, such as flowering and initial cone development (June, July), the temperature was lower in 2018 than in 2016 and 2017, and precipitation was higher (Figure 1). This region is at a low latitude, compared to Europe's major hop producing regions. In lower temperature years, plant growth conditions are closer to those observed at higher latitudes, where hops have better general growing conditions [2,8]. In several studies, it has been shown that the growing region, in general, has a great influence on the performance of hop plants [11,13,29,31–34]. It is also known that environmental variables can affect the secondary metabolism of plants and, therefore,

the accumulation of phenolic compounds [35,36]. Although plant vigor had a marked effect on tissue nutrient concentration [24], its effect on total phenols in hop coves was reduced.

The average content of total phenols in hop cones of the Nugget cultivar did not vary significantly between the plots of different plant vigour. The stress affecting plant growth and yield in the low vigour plots did not influence total phenols in the cones. A previous study analysing these plots [21] has shown that the plants appeared with excessive levels of Fe and Mn in the leaves, which may indicate poor soil aeration, probably caused by a deficient spatial water distribution along the rows by the flooding irrigation system. The soil texture in these plots did not seem to be different enough to create a gradient effect. Phenols significantly decreased with liming treatment. Likewise, Zu et al. [37] found a decrease in the flavonoid content of *Panax notoginseng* with calcium and lime application under cadmium stress. Although calcium seems to have an inhibitory effect on important enzymes in the phenolic pathway, it seems that the greater amount of cadmium in the roots inhibited the absorption of calcium and influenced flavonoid content. Unfortunately, with the data collected, it was not possible to identify the stress factors that caused the reduction in the content of phenols in the limed plots.

The foliar sprays did not influence significantly the content of total phenols in hop cones. To the best of our knowledge, results from hop cones have not yet been reported from experiments using foliar sprays. Foliar sprays, including those containing seaweed extracts, usually tend to increase the content of total phenols in plant tissues [17,38–40]. However, some studies have also reported an absence of a significant response to the application of this kind of products [41,42]. Of the cultivars, Nugget showed lower average values of total phenols in comparison to Cascade or Columbus if the two years were taken into account. From the samples selected for phenolic characterization, Nugget presented slightly higher values of total phenolic compounds but, in this case, just the samples with higher phenol content from the first year were characterized.

Previous studies have also shown significant differences in total phenols when different hop cultivars were compared [29,43,44]. The phenols content seems to depend on the cultivar and, in general, low molecular weight phenols are found in greater amounts in aroma cultivars, as the increase in alpha acid content seems to be achieved at the expense of the phenol content [4]. This seems to be true for Cascade, which showed significantly lower levels of alpha acid content, but not for Colombus, which was similar to Nugget, both presenting significantly higher levels of alpha acid content in comparison to Cascade [22]. Overall, the year average values found in this study ranged from 11.9 to 21.2 mg g^{-1} and were of similar magnitude to those reported by Kowalczyk et al. [45], varying between 16.2 and 25.5 mg g^{-1} (water extraction, followed by the Folin–Ciocalteau method). Lower values of 7.12 $\pm$ 0.09 mg GAE g^{-1} were reported by Keskin et al. [46] (methanol extraction, followed by the Folin–Ciocalteau method). These results emphasize the potential of the region to grow the cultivars Cascade and Columbus, along with the well-established Nugget.

4.2. PCA and Correlation Analysis

PCA and correlation analysis indicate a significant and negative association between total phenols and Zn concentrations in the cones. The results also indicate a negative influence of Cu, N and Fe in the accumulation of total phenols in the cones. Hop is a species particularly sensitive to Zn deficiency, affecting plant growth and cone production [47]. In this case, an association of Zn with plant vigour was not found, but higher concentrations of Zn, Cu and Fe were previously reported for these plots, and the result associated with poor soil aeration [21].

Enhanced absorption of Zn and Cu was also noticed in industrial hemp (*Cannabis sativa subsp. Sativa*) with higher irrigation level, with Zn showing higher mobility to aerial tissues [48]. The results of correlation analysis also showed significant and positive correlation between cone Cu and Fe, and cone Zn and Cu. Regarding Fe, the high levels previously reported in soil and plants [21], may have contributed to lowering total phenol concentrations. Zn, Fe and Cu do not seem to be important nutrients in phenolic biosynthe-

sis, and they may interfere negatively with other nutrients that provide co-factors for many enzymes of the flavonoid pathway [35].

Regarding N, its supplementation has been negatively associated with the phenolic composition of plant tissues in several crops [49,50], and associated with plant growth particularly in sensitive species to soil N availability [51]. In accordance with the protein competition model (PCM), since phenols and proteins compete for a common precursor, conditions that increase plant growth may reduce the concentration of total phenols [51]. Phenols are secondary metabolites synthesized through the shikimate pathway in which the amino acid phenylalanine is released, and this amino acid is a common precursor of phenylpropanoids and protein synthesis [35,51].

4.3. Phenolic Compounds Identification and Quantification

The phenolic compounds identified were mainly flavonols (quercetin and kaempferol) and phenolic carboxylic acids (*p*-coumaric and caffeic acids), which represent a minor fraction of the polyphenols that can be found in hop cones [5,7]. The result might be due to the in-water extraction method, which while suitable for many applications, is less efficient than the hydroalcoholic extraction method, particularly on hop prenylated flavonoids detection, which are lipophilic compounds [45]. The phenolic profile of *H. lupulus* is in accordance with those previously reported for bracts [7], leaves [52] and cones [53,54] and also for leaves, stem and roots of *H. japonicus* Siebold and Zucc [55]. The identification of peaks 8 ($[M-H]^-$ at *m/z* 609), 9 ($[M-H]^-$ at *m/z* 463), 11 ($[M-H]^-$ at *m/z* 593), and 12 ($[M-H]^-$ at *m/z* 447), quercetin-3-*O*-rutinoside, quercetin-3-*O*-hexoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, respectively, was performed by comparison of their retention time, UV spectra, and mass fragmentation patterns with the available commercial standards. Three caffeoylquinic acid derivatives were tentatively identified regarding the phenolic acid groups, peaks 1, 4, and 5 (3-*O*-, 4-*O*-, and 5-*O*-caffeoylquinic acids, respectively).

According to Clifford et al. [56,57], peaks 1 and 5 present a major ion MS^2 fragment at *m/z* 191, whereas peak 4 presents at *m/z* 173 an abundance of 100%, indicating the connection 4-*O*- position in the molecule. The organization of the three peaks, besides the major abundant fragments, was performed according to the hierarchical keys developed by Clifford et al. [56,57]. The two 3-*p*-coumaroylquinic acids found (peaks 2 and 3, *cis* and *trans*, respectively) were also assigned using the same hierarchical keys developed by Clifford et al. [56,57] the base peak at *m/z* 163 is for 3-*p*-coumaroylquinic acids. Since both peaks presented the same chromatographic characteristics, they were assigned as *cis* and *trans* isomers. Tanaka et al. [7] have also reported the same phenolic acids in the bracts of hop plants and Choi et al. [55] in the leaves, stem and roots of *Humulus japonicus* Siebold and Zucc. Finally, two *O*-glycosylated quercetin derivatives and two *O*-glycosylated kaempferol derivatives were also tentatively identified in the hop cones, peaks 6 and 10, and peaks 7 and 13, respectively. The tentative identification of these four peaks was performed based on those previously described in *H. lupulus* samples [7,52].

The hop cones of the less vigorous plants of the Nugget cultivar were higher in quercetin and kaempferol, whereas in the hops from the more vigorous plants, the kaempferol flavonoids and caffeic acids were found in small concentrations or were not even detectable. Environmental variables such as light exposure and temperature can significantly influence the accumulation of quercetin and kaempferol compounds in plant tissues [35]. Galieni et al. [58] have also found an increase in caffeic acid and other phenolic compounds in *Latuca sativa* L. grown under drought stress and an increase in cell wall lignification as a tolerance response.

In these experiments, the increased levels of phenolic compounds in less vigorous plants are probably a response to the environmental stress affecting plants' growth. The plants treated with foliar sprays presented slightly lower values of phenolic compounds. Similarly, Xu and Leskovar [42] did not find any effect of applying a seaweed extract on flavonoid content in spinach. Hop cones of plants on limed soil presented a significantly higher concentration of kaempferol-3-*O*-(2-rhamnosyl)-rutinoside and 4-*O*-caffeoylquinic

acid though not significantly. Likewise, Ngadze et al. [59] found an increase in caffeic acid content in potato (*Solanum tuberosum* L.) as a response to Ca applications.

As far as we know, no studies have reported the phenolic composition of hop cones after liming. Cascade stood out from the other cultivars, showing higher concentrations in quercetin and kaempferol compounds and lower in *p*-coumaric acids. Similarly, Almeida et al. [60] reported isoquercitrin followed by quercetin as the major phenolic compounds found in extracts of Cascade hops grown in Brazil. Santagostini et al. [61] identified quercetin-3-*O*-malonylglucoside and kaempferol-3-*O*-malonylglucoside compounds for the first time in Cascade hop. These compounds were also identified for the cultivars used in this study. In agreement with the present results, other studies [29,43] also showed significant differences in phenolic composition of different cultivars of hop, which probably was due to the potential influence of genetic factors on agronomic and biochemical traits [62].

5. Conclusions

Total phenols in hop cones were influenced significantly by most of the experimental factors (plant vigour, foliar treatment, liming, cultivar, plot and year) under study. However, in this study, foliar sprays and liming were among the factors that least influenced the measured variables (total phenol, nutrient concentration, and phenolic composition). The year, which represents the joint action of several environmental variables (temperatures, rainfall, relative humidity, etc.) resulted as the most important factor for the phenols accumulation between plants of different vigour, subject to different foliar treatments and grown in different plots and between different cultivars. Nugget showed significantly lower average values of total phenols than Cascade or Colombus cultivars if the two years were taken into account. The high levels of Zn in hop cones seemed to be associated with lower phenol content in the hop cones. The phenolic compounds identified were mainly flavonols (quercetin and kaempferol) and phenolic carboxylic acids (*p*-coumaric and caffeic acids). The less vigorous plants showed higher levels of quercetin and kaempferol in hop cones. The plants treated with foliar sprays (nutrient-rich and algae-rich foliar spray) presented slightly lower values of phenolic compounds, and plants on limed soil were notably higher in kaempferol-3-*O*-(2-rhamnosyl)-rutinoside. Cascade stood out from the other cultivars, showing higher concentration in quercetin and kaempferol compounds and lower concentration in *p*-coumaric acids. The phenolic compounds quercetin-3-*O*-malonylglucoside and kaempferol-3-*O*-malonylglucoside, reported previously in other studies for the first time in Cascade, were present in this study in Cascade, Columbus and Nugget. This study showed that most of the analysed compounds can vary in opposite directions with agro-environmental variables, making it difficult to recommend a coherent strategy to farmers without a well-defined target for the use of hop cones. It should also be noted that hop gardens are usually contaminated with viruses, especially old plantations, as may have been the case in the Nugget plots, which may have influenced the results of the experimental factors.

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Essential Oil Chemotypes of Four Vietnamese *Curcuma* Species Cultivated in North Alabama

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Abstract: *Curcuma* (turmeric) species are important culinary and medicinal plants, and the essential oils of *Curcuma* rhizomes have demonstrated promising pharmacological properties. The essential oils (EOs) of *Curcuma* species possess a wide variety of pharmacological properties, including anti-inflammatory, anticancerous, antiproliferative, hypocholesterolemic, antidiabetic, antirheumatic, hypotensive, antioxidant, antimicrobial, antiviral, antithrombotic, antityrosinase, and cyclooxygenase-1 (COX-1) inhibitory activities, among others. *Curcuma* oils are also known to enhance immune function, promote blood circulation, accelerate toxin elimination, and stimulate digestion. *C. longa* (turmeric) and *C. zedoaria* (zedoary) are the most extensively studied species of *Curcuma* due to their high commercial value. There is some interest in expanding the cultivation of *Curcuma* species to the southern regions in North America where the climate is favorable. The purpose of this work was to examine the rhizome essential oil composition of four species of *Curcuma* (*C. aromatica*, *C. caesia*, *C. longa*, *C. zanthorrhiza*) that were obtained from Vietnam and cultivated in North Alabama. The rhizome essential oils were obtained by hydrodistillation and analyzed by gas chromatographic techniques. The essential oils of *C. aromatica* were dominated by curzerenone (14.7–18.6%), germacrone (10.7–14.7%), 1,8-cineole (5.2–11.7%), and an unidentified component (8.7–11.0%). The major components in *C. longa* rhizome oil were *ar*-turmerone (8.3–36.1%), α-turmerone (12.7–15.2%), β-turmerone (5.0–15.4%), α-zingiberene (4.6–13.9%), and β-sesquiphellandrene (4.6–10.0%). The essential oils of *C. caesia* and *C. zanthorrhiza* were rich in curzerenone, curdione, and germacrone. These adapted turmeric varieties in North Alabama have potential use for medical purposes and medicinal plant oil market demands in the U.S.

Keywords: turmeric; *Curcuma aromatica*; *Curcuma caesia*; *Curcuma longa*; *Curcuma zanthorrhiza*; chemical composition; enantiomeric distribution; chiral

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1. Introduction

There are currently 93 recognized species of *Curcuma* L. (Zingiberaceae) [1]. These perennial rhizomatous herbs originated in subtropical and tropical areas of Asia, Australia, and South America [2], and a number of *Curcuma* species are cultivated in large scales in India, Nepal, Pakistan, Bangladesh, Indonesia, Malaysia, and Thailand [3]. *Curcuma* species are herbaceous perennial herbs with tuberous rhizomes (underground stems). Among them, some important species such as *Curcuma amada* Roxb. (mango ginger), *Curcuma angustifolia* Roxb. (wild arrowroot), *C. aromatica* Salisb. (wild turmeric), *Curcuma caesia* Roxb. (black turmeric), *Curcuma decipiens* Dalzell, *Curcuma kwangsiensis* S. G. Lee & C. F. Liang, *Curcuma longa* L., *Curcuma montana* Roxb., *Curcuma ochrorhiza* Valeton, *Curcuma pierreana* Gagnep., *Curcuma roscoeana* Wall., and *Curcuma zedoaria* (Christm.) Roscoe (zedoary) have economical value as they are used in medicine, cosmetics, and both the floricultural and culinary industries [4]. Turmeric is mainly used for culinary, medicinal, and aromatic purposes. Its

rhizomes are the ancient colorful spice source and have a bitter and pungent taste and a pepper-like aroma. Turmeric is also known as the "Golden Spice of India" [5] or "Kitchen Queen" [6]. For example, it has been used in curries in India; in Japan and Korea it is popularly served as a herbal tea; and it is used as a preservative and a coloring agent in mustard sauce, cheese, butter, and chips in the western world [7]. Curcuminoids and the essential oil of turmeric are associated with a myriad of medicinal, culinary and industrial properties of curcuma species [8], which are derived from the underground plant part, rhizomes (actually the stem), which are tuberous, with a rough and segmented skin. The primary rhizome is known as "mother rhizome" or bulb, and is pear-shaped in the center (Figure 1). The branches of mother rhizomes are the secondary rhizomes, called lateral or "finger rhizomes" [9].

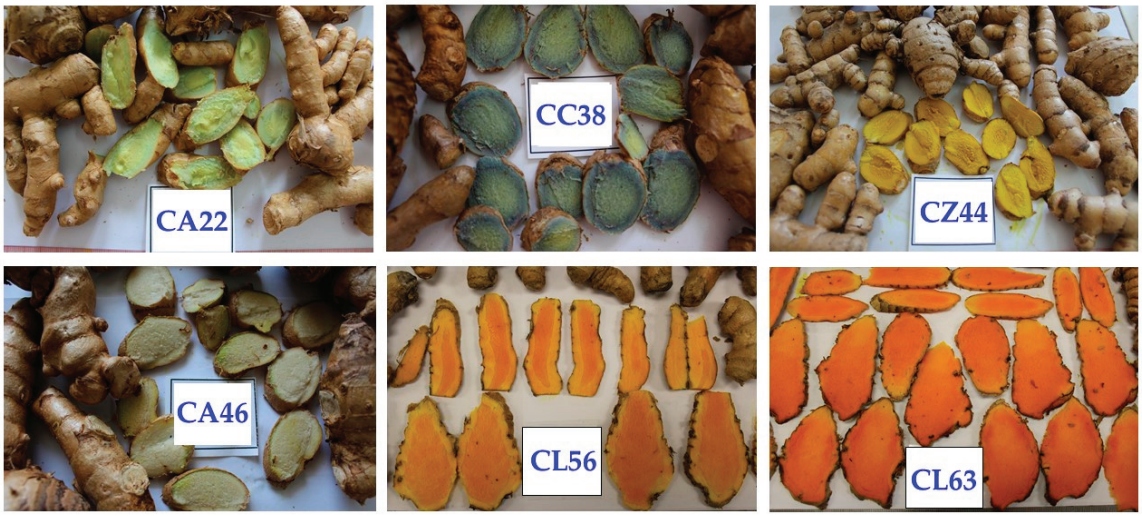

Figure 1. Rhizomes of Vietnamese *Curcuma* species cultivated in North Alabama: *Curcuma aromatica* (green rhizome, CA22; white rhizome, CA46), *Curcuma caesia* (black rhizome, CC38), *Curcuma zanthorrhiza* (lime rhizome, CZ44), *Curcuma longa* (yellow rhizome, CL56; red rhizome, CL63). Photographs taken by Lam Duong.

Though turmeric has been known for its multiple uses for over 4000 years in India [10], its use as a medicinal and health supplement in the United States is of recent origin. The interest in turmeric in the U.S. has been increasing over the past two decades mainly due to a large number of scientific publications on its medicinal benefits [8]. To meet the growing demand for turmeric, the U.S. imports 90% of its market demand mainly from India. The U.S. import market was estimated at USD 87.28 million in 2018 [11]. The large market for turmeric in the United States suggests that there is opportunity for cultivation of turmeric in this country provided varieties with high curcumin yield and desirable essential oil composition are available.

Curcuma aromatica Salisb. (wild turmeric) is found naturally in South Asia, including southern China, Bhutan, Myanmar, India, Nepal, Sri Lanka [12], and Vietnam [13], and is widely cultivated in China, India, and Japan [14]. The plant is used in traditional medicines throughout its range for its wound-healing, anti-inflammatory, anti-tumor, immunomodulatory, antimicrobial effects and as an antidote for snake venom [15–17]. The rhizome essential oils are generally dominated by camphor, curzerenone, germacrone, curdione, and 1,8-cineole [14].

Curcuma caesia Roxb. (black turmeric) grows wild in northeastern and central India, Malaysia, Thailand, and Indonesia [14,18]. The rhizome of *C. caesia* has been used as a traditional medicine to treat leprosy, bronchitis, asthma, cancer, epilepsy, fever, wounds, im-

potence, fertility, vomiting, and pain [19]. *Curcuma caesia* is considered to be endangered in its native range in India [18], however, it has been underexplored in terms of cultivation and commercialization [20]. The major components in the rhizome essential oil of *C. caesia* from northeastern India were camphene, 1,8-cineole, camphor, borneol, (*E*)-β-caryophyllene, and *ar*-turmerone, which defined two chemotypes, a camphor/*ar*-turmerone chemotype and a 1,8-cineole/(*E*)-β-caryophyllene chemotype [20].

Curcuma longa L. (turmeric) is cultivated worldwide, especially in tropical countries in Asia, Australia, and the Neotropics [9]. It is a well-known medicinal agent and culinary ingredient. In addition to curcumin and other non-volatile curcuminoids, the essential oil of turmeric has been employed in the treatment of various maladies in humans and animals [21]. Turmeric essential oils are made up of hundreds of components and the major components, however, are α-turmerone, β-turmerone, *ar*-turmerone, β-sesquiphellandrene, α-zingiberene, germacrone, terpinolene, *ar*-curcumene, and α-phellandrene [3,22].

Curcuma zanthorrhiza Roxb. (Javanese turmeric) is often referred to in the literature as *Curcuma xanthorrhiza* Roxb., however, that name is not recognized by World Flora Online [23]. The plant is native to Indonesia, although is also cultivated in Malaysia, the Philippines, Thailand, Vietnam, and to a lesser extent in China, India, Japan, and South Korea [24]. Traditional medicinal uses of the plant include treatment for stomach illness, liver ailments, constipation, bloody diarrhea, dysentery, arthritis, rheumatism, fevers, hemorrhoids, vaginal discharge, and skin eruptions [24].

As part of our research program investigating potential cultivation of *Curcuma* in Alabama, *Curcuma aromatica* (both green- and white-colored rhizomes), *C. caesia* (black-colored rhizome), *C. zanthorrhiza* (lime-green rhizome), and *C. longa* (both yellow-, and red-colored rhizomes), obtained from Vietnam, were cultivated in North Alabama (Figure 1). The rhizome essential oils were obtained by hydrodistillation and analyzed by gas chromatographic methods. Both the "mother" or main rhizomes as well as the "daughter" or finger rhizomes were obtained and analyzed. The six *Curcuma* varieties used in this study were selected out of 64 genotypes according to three criteria: high yield but low curcuminoid content (variety, CL56), high yield but no curcumin content (CA22, CA46, CC38, and CZ44), and high yield and high curcumin content (CL63) based on unpublished data by the authors, Lam Duong and S.R. Mentreddy at Alabama A&M University.

2. Materials and Methods

2.1. Plant Material

The six *Curcuma* varieties used in this study were collected by Lam Duong from various locations in Vietnam: CA22 (Quang Nam province), CA46 (Gia Lai province), CC38 (Nghe An province), CL56 (Bac Giang province), CL63 (Quang Tri province), and CZ44 (Gia Lai province). The *Curcuma* rhizomes varieties were initially planted in a glass greenhouse at Alabama A&M University (Normal, AL, USA) and subsequently cultivated at the Alabama A&M Winfred Thomas Agricultural Research Station (Hazel Green, AL, USA, 34′89″ N, 86′56″ W) as previously described for *C. longa* cultivation [9]. Each of the fresh *Curcuma* rhizomes were collected on 18 March 2021, and stored at −20 °C until processed. The mother and daughter rhizomes were, separately, chopped and hydrodistilled using a Likens–Nickerson apparatus for 4 h (see Table 1).

Table 1. *Curcuma* rhizome hydrodistillation yields.

Sample Code	Rhizome Color	*Curcuma* Species	Mother Mass (g) Daughter Mass (g)	Essential Oil Yield (mg)	% Yield	Essential Oil Color
CA22	Green	*C. aromatica*	58.72 101.27	401.9 571.4	0.68 0.56	pale yellow pale yellow
CA46	White	*C. aromatica*	48.37 85.16	382.3 346.3	0.79 0.41	pale yellow pale yellow
CC38	Black	*C. caesia*	131.92 47.14	981.2 531.6	0.74 1.13	pale yellow pale yellow
CL56	Yellow	*C. longa*	73.70 85.76	431.7 527.0	0.59 0.61	colorless colorless
CL63	Red	*C. longa*	40.61 66.49	372.4 368.1	0.92 0.55	colorless colorless
CZ44	Lime	*C. zanthorrhiza*	64.96 79.94	700.2 728.7	1.08 0.91	colorless colorless

2.2. Gas Chromatographic Analyses

The *Curcuma* rhizome essential oils were analyzed by gas chromatography—mass spectrometry (GC-MS) as previously reported [3]: Shimadzu GCMS-QP2010 Ultra (Shimadzu Scientific Instruments, Columbia, MD, USA), electron impact (EI) mode (70 eV), scan range 40–400 m/z, scan rate 3.0 scans/s; ZB-5ms GC column (60 m length × 0.25 mm diameter × 0.25 μm film thickness) (Phenomenex, Torrance, CA, USA), He carrier gas, 208.3 kPa head pressure, flow rate 2.0 mL/min, injector temperature 260 °C, ion source temperature 260 °C, oven temperature program 50 °C to 260 °C at 2 °C/min then held at 260 °C for 5 min; 0.3 μL of 5% *w*/*v* solutions in CH_2Cl_2 were injected, split ratio 1:24. Essential oil components were identified by comparison of MS fragmentation and retention index (RI) with those provided in the databases [25–28].

Gas chromatography with flame ionization detection (GC-FID) was carried out as previously reported [29]: Shimadzu GC 2010 (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with flame ionization detector, ZB-5 capillary column (60 m × 0.25 mm i.d.; film thickness 0.25 μm) (Phenomenex, Torrance, CA, USA); oven temperature programmed as above for GC-MS; injector and detector temperatures 260 °C; He carrier gas, flow rate 1.0 mL/min; 0.3 μL of 5% *w*/*v* solution in CH_2Cl_2 were injected, ratio 1:31. The percent compositions of the essential oils were calculated from peak areas with quantification using the external standard method; calibration curves of representative compounds from each class were used for quantification.

Chiral GC-MS was carried out as previously reported [29]: Shimadzu GCMS-QP2010S instrument (Shimadzu Scientific Instruments, Columbia, MD, USA), Restek B-Dex 325 column (Restek Corporation, Bellefonte, PA, USA); oven temperature program 50 °C to 120 °C at 1.5 °C/min, then to 200 °C at 2.0 °C/min; 0.1 μL of 5% *w*/*v* solutions in CH_2Cl_2 were injected, with a split ratio of 1:25. The enantiomers were determined by comparison of retention times with authentic samples obtained from Sigma-Aldrich (Milwaukee, WI, USA) and the relative enantiomer percentages were calculated from peak integration.

2.3. Statistical Analysis

Analysis of variance was conducted by one-way ANOVA followed by the Tukey test using Minitab® 18 (Minitab Inc., State College, PA, USA). Differences at $p < 0.05$ were considered to be statistically significant. For the agglomerative hierarchical cluster (AHC) analysis, the 12 essential oil compositions were treated as operational taxonomic units (OTUs), and the concentrations (percentages) of 18 of the most abundant essential oil components (curzerenone, curdione, germacrone, *ar*-turmerone, 1,8-cineole, α-turmerone, unidentified (RI = 1778), β-turmerone (=curlone), β-sesquiphellandrene, α-zingiberene, *iso*-curcumenol, curcumenone, *trans*-β-elemene, *ar*-curcumene, β-pinene, curcumenol, cam-

phor, and curzerene) were used to determine the chemical associations between the *Curcuma* rhizome essential oil samples using XLSTAT Premium, version 2018.1.1.62926 (Addinsoft, Paris, France). Similarity was determined using the Pearson correlation, and clustering was defined using the unweighted pair-group method with arithmetic mean (UPGMA).

3. Results and Discussion

3.1. Chemical Composition of Curcuma Rhizome Essential Oils

The fresh rhizome samples were hydrodistilled to give colorless or pale-yellow essential oils in yields ranging from 0.41% to 1.13% (Table 1). The chemical composition of the *Curcuma* rhizome essential oils are compiled in Table 2. Gas chromatograms of each Curcuma variety are shown in Supplementary Figure S1.

The essential oils from the green-colored mother and daughter rhizomes of *C. aromatica* (CA22) were dominated by curzerenone (18.6% and 14.7%, respectively), germacrone (14.7% and 10.7%, respectively), 1,8-cineole (11.7% and 6.6%, respectively), and an unidentified sesquiterpenoid (RI 1778, Supplementary Figure S2) (9.0 and 8.7%, respectively). Similarly, the white-colored mother and daughter rhizomes (*C. aromatica*, CA46) were dominated by the same components, curzerenone (14.9% and 14.8%), germacrone (14.5% and 12.5%), 1,8-cineole (10.2% and 5.2%), and the unidentified compound (RI 1778) (11.0% and 10.3%). The rhizome essential oil composition of *C. aromatica* show wide variation depending on geographical location [14]. For example, camphor was found to be a major component of *C. aromatica* rhizome essential oils from India (18.8–32.3%), whereas 8,9-dehydro-9-formylcycloisolongifolene (2.7–36.8%) was a dominant compound in the essential oils from China. Camphor was relatively minor in *C. aromatica* cultivated in North Alabama in this work (1.4–2.5%) and 8,9-dehydro-9-formylcycloisolongifolene was not observed. Curzerenone, germacrone, and 1,8-cineole, however, are relatively concentrated in Indian *C. aromatica* rhizome essential oils [14]. The rhizome essential oil of *C. aromatica* from Thailand showed camphor (26.9%), *ar*-curcumene (23.2%), and xanthorrhizol (18.7%) as the major components [30], whereas the rhizome essential oil from *C. aromatica* cultivated in Japan revealed β-turmerone (32.2 and 44.0%), 1,8-cineole (7.5 and 25.3%), and germacrone (4.6 and 9.6%) to be major compounds [31]. Notably, curzerenone, 8,9-dehydro-9-formylcycloisolongifolene, *ar*-curcumene, and xanthorrhizol were not detected in the essential oils from Japan. A recent examination of *C. aromatica* from different regions of eastern and southern India revealed relatively low concentrations of curzerenone (0.0–1.2%), but high concentrations of xanthorrhizol (8.8–24.4%), camphor (4.1–18.1%), germacrone (3.5–21.9%), neocurdione (5.8–14.6%), and 1,8-cineole (3.7–11.9%) [32].

The black-rhizome (*C. caesia*, CC38) essential oil, on the other hand, was rich in curzerenone (26.1% and 29.1%), curdione (28.7% and 35.6%), as well as *iso*-curcumenol (6.5% and 5.6%), for the mother and daughter rhizomes, respectively. In contrast, *C. caesia* rhizome essential oil from north India showed 8,9-dehydro-9-formylcycloisolongifolene, (11.7%), camphor (6.1%), 1,8-cineole (6.0%), and β-elemene (5.2%, reported as β-germacrene) as major components [33]. Neither curzerenone, curdione, nor *iso*-curcumenol were reported in the essential oil from north India, and 8,9-dehydro-9-formylcycloisolongifolene was not found in the essential oil from North Alabama. Note that curzerenone was determined to be artificially elevated in *C. caesia* essential oil due to the Cope rearrangement of furanodienone [34].

Table 2. Chemical compositions of the rhizome (mother and daughter) essential oils of *Curcuma* species from Vietnam, cultivated in North Alabama.

RI_{calc}	RI_{db}	Compound	*Curcuma aromatica* (Green Rhizome)		*Curcuma aromatica* (White Rhizome)		*Curcuma caesia* (Black Rhizome)		*Curcuma zanthorrhiza* (Lime Rhizome)		*Curcuma longa* (Yellow Rhizome)		*Curcuma longa* (Red Rhizome)	
			CA22 (M)	CA22 (D)	CA46 (M)	CA46 (D)	CC38 (M)	CC38 (D)	CZ44 (M)	CZ44 (D)	CL56 (M)	CL56 (D)	CL63 (M)	CL63 (D)
807	802	2-Hexanol	0.2	0.1	—	—	0.1	—	—	—	—	—	—	—
888	889	2-Heptanone	—	0.1	0.1	0.1	tr	tr	tr	tr	tr	tr	—	—
897	900	2-Heptanol	0.2	0.2	—	—	0.3	—	—	0.1	0.2	0.1	—	—
918	923	Tricyclene	tr	tr	tr	tr	—	—	0.1	tr	—	—	—	—
926	925	α-Thujene	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
933	933	α-Pinene	0.5	1.0	0.5	0.9	tr	0.1	0.6	0.6	0.2	0.1	0.1	0.1
949	950	Camphene	0.3	0.4	0.2	0.3	tr	tr	1.4	0.7	tr	—	tr	—
962	960	Benzaldehyde	—	—	—	—	tr	tr	—	—	tr	—	tr	tr
972	972	Sabinene	0.2	0.3	0.2	0.2	tr	tr	0.1	0.1	0.1	tr	tr	tr
979	**978**	**β-Pinene**	**2.4**	**6.2**	**2.5**	**5.9**	**0.3**	**0.5**	**2.7**	**3.4**	**0.2**	**0.5**	**tr**	**0.2**
980	979	2-Methyl-2-hepten-4-one	—	—	—	—	—	—	—	—	—	—	tr	—
984	986	6-Methyl-5-hepten-2-one	—	—	—	—	—	—	—	—	—	—	tr	tr
986	986	*p*-Menth-3-ene	—	—	tr	tr	tr	tr	—	—	—	—	—	—
989	989	Myrcene	0.2	0.3	0.2	0.2	tr	tr	0.3	0.2	0.1	0.1	0.1	0.1
989	989	2-Octanone	—	—	—	—	tr	—	—	—	—	—	—	—
1002	1004	2-Octanol	—	—	—	—	0.1	—	—	—	—	—	—	—
1007	1007	α-Phellandrene	—	—	—	—	—	—	—	—	tr	tr	0.1	0.1
1009	1009	δ-3-Carene	—	—	—	—	—	—	—	—	tr	tr	tr	tr
1017	1017	α-Terpinene	—	—	—	—	—	—	—	—	0.1	tr	0.1	0.1
1024	1025	*p*-Cymene	0.1	0.1	0.1	tr	tr	tr	tr	tr	0.1	tr	0.1	0.1
1029	1030	Limonene	0.6	0.6	0.5	0.5	0.1	tr	0.7	0.3	0.3	0.1	0.1	0.1
1033	**1032**	**1,8-Cineole**	**11.7**	**6.6**	**10.2**	**5.2**	**2.0**	**0.5**	**7.2**	**2.5**	**13.1**	**3.3**	**3.5**	**1.4**
1035	1034	(*Z*)-β-Ocimene	—	—	—	—	—	—	—	—	—	tr	—	—
1038	1041	2-Heptyl acetate	—	—	—	—	tr	tr	tr	tr	—	tr	—	—
1045	1045	(*E*)-β-Ocimene	—	—	—	—	—	—	—	—	—	tr	—	—
1057	1057	γ-Terpinene	—	—	—	—	—	—	—	—	tr	tr	tr	tr
1085	1086	Terpinolene	—	—	—	—	—	tr	tr	tr	1.0	1.1	2.3	2.6
1090	1093	*p*-Cymenene	—	—	—	—	—	—	—	—	—	—	0.1	0.1
1090	1090	2-Nonanone	0.3	0.5	0.2	0.4	0.7	0.6	0.4	0.4	0.1	0.1	—	—
1099	1099	Linalool	0.1	0.2	tr	0.1	0.2	0.1	0.5	0.3	0.1	tr	tr	tr
1105	1105	2-Nonanol	0.3	0.6	—	—	2.3	—	—	—	0.3	0.2	tr	tr
1138	1139	Nopinone	—	—	—	tr	—	—	—	—	—	—	—	—
1142	1141	(*E*)-Myroxide	—	—	—	—	—	—	—	—	—	tr	0.1	tr
1146	**1145**	**Camphor**	**1.8**	**2.5**	**1.4**	**2.1**	**tr**	**tr**	**7.6**	**3.6**	**—**	**—**	**—**	**—**
1146	1146	Myrcenone	—	—	—	—	—	—	—	—	—	—	tr	tr
1155	1156	Camphene hydrate	0.2	0.3	0.1	0.2	—	—	0.2	0.1	—	—	—	—
1162	1164	Pinocarvone	—	tr	tr	tr	—	—	—	—	—	—	—	—
1164	1165	*iso*-Borneol	0.4	0.4	—	—	—	—	—	0.6	—	—	—	—
1170	1170	δ-Terpineol	0.1	tr	0.1	tr	tr	—	—	—	0.1	tr	—	—
1174	1173	Borneol	0.2	0.3	—	—	—	—	—	—	—	—	—	—

Table 2. *Cont.*

RI_{calc}	RI_{db}	Compound	*Curcuma aromatica* (Green Rhizome)		*Curcuma aromatica* (White Rhizome)		*Curcuma caesia* (Black Rhizome)		*Curcuma zanthorrhiza* (Lime Rhizome)		*Curcuma longa* (Yellow Rhizome)		*Curcuma longa* (Red Rhizome)	
			CA22 (M)	CA22 (D)	CA46 (M)	CA46 (D)	CC38 (M)	CC38 (D)	CZ44 (M)	CZ44 (D)	CL56 (M)	CL56 (D)	CL63 (M)	CL63 (D)
1175	1176	*cis*-Pinocamphone	—	—	—	—	tr	—	—	—	—	—	—	—
1180	1180	Terpinen-4-ol	0.3	0.2	0.3	0.2	tr	tr	0.2	0.1	0.3	0.1	tr	tr
1184	1188	4′-Methylacetophenone	—	—	—	—	—	—	—	—	—	tr	tr	tr
1187	1186	*p*-Cymen-8-ol	—	—	—	—	—	—	—	—	0.2	0.1	0.3	0.3
1189	1290	Indole	—	—	tr	tr	tr	tr	—	—	—	—	tr	—
1191	1190	2-Decanone	—	tr	tr	tr	0.1	0.1	tr	tr	tr	tr	—	—
1195	1195	α-Terpineol	0.7	0.5	0.7	0.4	0.1	tr	0.4	0.2	1.3	0.3	tr	0.1
1203	1203	*p*-Cumenol	—	—	—	—	—	—	—	—	—	—	tr	tr
1206	1205	Verbenone	—	—	tr	tr	tr	tr	—	—	—	—	—	—
1209	1211	2-Decanol	—	—	—	—	0.1	—	—	—	—	—	—	—
1232	1233	2-Nonyl acetate	—	—	—	—	tr	tr	—	—	—	—	—	—
1243	1242	Carvone	tr	tr	tr	tr	tr	tr	—	—	—	—	—	—
1274	1274	Cyclooctyl acetate	—	—	—	—	0.1	0.1	—	—	—	—	—	—
1291	1293	2-Undecanone	0.1	0.1	tr	0.1	0.2	0.2	0.1	0.1	0.1	0.1	tr	tr
1310	1317	2-Undecanol	—	—	—	—	0.1	—	—	—	—	—	—	—
1331	1334	Bicycloelemene	—	—	—	—	tr	tr	—	—	—	—	—	—
1334	1335	δ-Elemene	0.2	0.4	0.2	0.3	0.2	0.2	0.2	0.3	tr	tr	—	—
1336	1339	Piperitenone	—	—	—	—	—	—	—	—	0.1	0.1	—	tr
1343	1344	Evodone	—	—	—	—	—	tr	—	—	—	—	—	—
1346	1348	α-Cubebene	—	tr	—	tr	—	—	—	—	—	—	—	—
1368	1367	Cyclosativene	—	tr	—	tr	—	—	—	—	—	—	tr	—
1374	1375	α-Copaene	—	tr	tr	tr	—	—	—	—	—	—	tr	—
1381	1383	*cis*-β-Elemene	0.1	0.3	0.1	0.2	0.2	0.1	0.1	0.2	tr	tr	—	—
1390	**1390**	***trans*-β-Elemene**	**2.7**	**4.9**	**2.4**	**3.7**	**2.9**	**2.0**	**2.6**	**3.5**	**0.3**	**0.4**	**—**	**0.1**
1393	1394	Sativene	—	—	—	—	tr	tr	tr	tr	—	—	—	—
1402	1405	Sesquithujene	—	—	—	—	—	—	—	—	0.1	0.2	0.1	0.1
1418	1417	(*E*)-β-Caryophyllene	0.4	1.4	0.4	1.1	0.9	0.8	0.5	1.1	0.1	0.3	1.2	2.1
1423	1425	α-Sinensal	—	—	tr	tr	tr	tr	tr	tr	—	—	—	—
1428	1427	γ-Elemene	0.3	0.5	0.3	0.4	0.3	0.2	0.3	0.5	0.1	0.1	—	tr
1432	1432	*trans*-α-Bergamotene	—	—	—	—	—	—	—	—	0.1	0.1	tr	tr
1440	1442	Guaia-6,9-diene	—	—	—	—	tr	tr	—	—	—	—	—	—
1442	1439	(*Z*)-β-Farnesene	—	—	—	—	—	—	—	—	—	—	tr	tr
1447	1447	*iso*-Germacrene D	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	—	—	—	—
1451	1452	(*E*)-β-Farnesene	0.1	0.3	0.1	0.2	0.1	0.1	0.1	0.1	0.5	0.5	0.4	0.3
1455	1454	α-Humulene	0.1	0.2	0.1	0.2	1.7	1.5	1.8	2.5	—	—	0.5	0.5
1459	1458	*allo*-Aromadendrene	tr	0.1	tr	0.1	—	—	tr	tr	—	—	—	—
1462	1462	α-Acoradiene	—	—	—	—	—	—	—	—	—	—	tr	tr
1472	1474	Selina-4,11-diene	—	—	—	tr	tr	tr	—	—	—	—	—	—
1473	1473	Dauca-5,8-diene	—	—	0.1	0.3	tr	tr	0.1	0.2	—	—	—	—
1474	1475	γ-Muurolene	0.1	0.3	—	—	tr	tr	—	—	—	—	—	—

Table 2. *Cont.*

RI_{calc}	RI_{db}	Compound	*Curcuma aromatica* (Green Rhizome)		*Curcuma aromatica* (White Rhizome)		*Curcuma caesia* (Black Rhizome)		*Curcuma zanthorrhiza* (Lime Rhizome)		*Curcuma longa* (Yellow Rhizome)		*Curcuma longa* (Red Rhizome)	
			CA22 (M)	CA22 (D)	CA46 (M)	CA46 (D)	CC38 (M)	CC38 (D)	CZ44 (M)	CZ44 (D)	CL56 (M)	CL56 (D)	CL63 (M)	CL63 (D)
1476	1480	β-Acoradiene	—	—	—	—	—	—	—	—	—	—	tr	tr
1480	1480	*ar*-Curcumene	1.6	3.1	1.8	2.5	—	—	1.9	2.3	3.5	3.6	2.6	2.4
1480	1480	Germacrene D	—	—	—	—	0.8	0.4	—	—	—	—	—	—
1484	1483	*trans*-β-Bergamotene	—	—	—	—	—	—	—	—	0.1	0.1	0.1	tr
1488	1489	β-Selinene	0.3	0.4	0.3	0.4	0.2	0.2	0.2	0.2	—	—	—	—
1494	**1493**	**Curzerene**	**1.2**	**2.0**	**1.4**	**1.9**	**3.3**	**1.8**	**2.4**	**3.9**	**—**	**—**	**—**	**—**
1495	1497	α-Selinene	0.4	0.5	0.2	0.2	—	0.1	0.1	0.1	—	—	—	—
1496	**1496**	**α-Zingiberene**	**—**	**—**	**—**	**—**	**—**	**—**	**—**	**0.3**	**11.4**	**13.9**	**3.4**	**4.6**
1497	1497	α-Muurolene	0.1	0.3	0.1	0.5	—	—	0.1	0.3	—	—	—	—
1504	1501	β-Dihydroagarofuran	—	—	—	—	0.1	0.1	—	—	—	—	—	—
1504	1502	*trans*-β-Guiaene	—	—	—	—	0.1	tr	tr	—	—	—	—	—
1505	1505	α-Bulnesene	0.1	0.2	—	—	—	—	—	—	—	—	—	—
1507	1504	*iso*-Daucene	—	—	—	—	0.1	0.1	—	—	—	—	—	—
1508	1508	β-Bisabolene	0.5	1.0	0.6	0.8	—	—	0.5	0.5	2.4	2.5	0.8	0.8
1513	1512	γ-Cadinene	0.1	0.2	0.1	0.1	—	—	0.1	0.1	—	—	—	—
1517	1519	Cubebol	0.2	0.4	—	—	—	—	—	—	—	—	—	—
1518	1518	δ-Cadinene	0.3	0.9	0.3	0.5	tr	0.1	0.3	0.6	—	—	—	—
1520	1518	7-*epi*-α-Selinene	—	—	—	—	—	—	—	tr	—	—	—	—
1521	1523	β-Guaiene	—	—	—	—	—	—	—	tr	—	—	—	—
1522	**1523**	**β-Sesquiphellandrene**	**1.1**	**2.5**	**1.6**	**1.9**	**—**	**tr**	**1.6**	**1.9**	**8.9**	**10.0**	**4.9**	**4.6**
1528	1528	(*E*)-γ-Bisabolene	—	—	—	—	—	—	—	—	0.2	0.2	0.2	0.2
1549	1549	α-Elemol	—	—	—	—	0.2	—	—	—	—	—	—	—
1555	1555	7-*epi*-*cis*-Sesquisabinene hydrate	—	—	—	—	—	—	—	—	0.5	0.4	0.2	0.1
1558	1560	Germacrene B	0.5	0.9	0.6	0.8	0.7	0.5	0.8	0.9	0.3	0.2	—	—
1560	1560	(*E*)-Nerolidol	—	—	—	—	—	—	—	—	0.1	tr	0.5	—
1578	1578	*ar*-Tumerol	—	—	—	—	—	—	—	—	0.3	0.2	0.8	0.5
1582	1587	Caryophyllene oxide	0.2	0.5	0.2	0.4	0.3	0.4	0.2	0.2	0.1	0.1	—	0.4
1590	1590	Globulol	0.2	0.3	—	—	0.3	—	—	—	—	—	—	—
1592	1591	7-*epi*-*trans*-Sesquisabinene hydrate	—	—	—	—	—	—	—	—	1.6	1.5	0.3	0.7
1592	1591	*cis*-β-Elemenone	—	—	0.2	0.1	—	—	0.2	—	—	—	—	—
1595	1594	Viridiflorol	0.7	0.2	0.2	0.2	0.1	0.2	0.1	0.3	—	—	—	—
1596	1596	*trans*-β-Elemenone	—	1.6	3.0	2.7	—	—	1.8	1.9	1.3	1.1	—	0.4
1600	1601	Humulene epoxide I	—	—	—	—	2.2	1.7	—	—	—	—	—	—
1600	1599	*anti*,*anti*,*anti*-Helifolen-12-al B	—	—	—	—	—	—	—	—	0.2	0.3	0.8	0.6
1602	**1603**	**Curzerenone**	**18.6**	**14.7**	**14.9**	**14.8**	**26.1**	**29.1**	**16.3**	**19.7**	**—**	**—**	**—**	**—**
1611	1613	Humulene epoxide II	—	—	—	—	—	0.3	0.3	0.3	—	—	—	—

Table 2. *Cont.*

RI_{calc}	RI_{db}	Compound	*Curcuma aromatica* (Green Rhizome)		*Curcuma aromatica* (White Rhizome)		*Curcuma caesia* (Black Rhizome)		*Curcuma zanthorrhiza* (Lime Rhizome)		*Curcuma longa* (Yellow Rhizome)		*Curcuma longa* (Red Rhizome)	
			CA22 (M)	CA22 (D)	CA46 (M)	CA46 (D)	CC38 (M)	CC38 (D)	CZ44 (M)	CZ44 (D)	CL56 (M)	CL56 (D)	CL63 (M)	CL63 (D)
1614	1610	*iso*-Curzerenone	—	—	—	—	0.2	0.3	—	—	—	—	—	—
1615	1615	Zingiberenol	0.7	0.8	0.7	0.8	—	—	—	—	2.9	2.6	0.9	1.2
1621	**1616**	***iso*-Curcumenol**	**4.0**	**3.1**	**3.7**	**3.4**	**6.5**	**5.6**	**2.6**	**0.8**	**0.6**	**0.6**	**—**	**—**
1630	1629	*iso*-Spathulenol	0.7	0.4	0.8	1.0	0.5	—	0.8	0.5	—	—	—	—
1632	—	Unidentified [a]	0.2	—	—	—	0.9	0.4	—	0.5	2.0	1.7	0.4	1.5
1636	—	Unidentified [a]	—	—	—	—	0.1	0.9	—	—	0.4	0.4	0.3	1.2
1643	1643	τ-Cadinol	0.3	0.4	0.4	0.7	—	—	—	0.3	—	—	—	—
1643	1644	*allo*-Aromadendrene epoxide	—	—	—	—	—	—	0.1	—	—	—	—	—
1646	1645	τ-Muurolol	0.3	0.6	—	—	—	—	—	—	—	—	—	—
1648	1651	α-Muurolol	—	—	—	—	—	—	—	0.1	—	—	—	—
1655	1655	α-Cadinol	0.3	0.4	0.4	0.4	—	—	0.7	1.1	—	—	—	—
1659	1656	β-Eudesmol	—	—	—	—	1.7	0.3	—	—	—	—	—	—
1663	**1664**	***ar*-Turmerone**	**—**	**0.2**	**0.3**	**0.4**	**—**	**—**	**—**	**—**	**8.3**	**12.6**	**36.1**	**31.3**
1667	**1668**	**α-Turmerone**	**—**	**—**	**—**	**—**	**—**	**—**	**—**	**—**	**12.7**	**14.1**	**15.2**	**13.0**
1688	1687	α-Bisabolol	—	0.4	—	—	—	—	0.3	—	0.4	0.4	0.2	0.2
1695	**1694**	**Germacrone**	**14.7**	**10.7**	**14.5**	**12.5**	**4.9**	**3.8**	**11.3**	**11.1**	**5.5**	**4.6**	**—**	**1.5**
1700	**1699**	**β-Turmerone (=Curlone)**	**—**	**—**	**—**	**0.2**	**—**	**—**	**—**	**—**	**5.0**	**6.9**	**15.4**	**13.0**
1714	1712	Curcuphenol	—	—	—	—	—	—	—	—	—	0.1	—	—
1718	**1713**	**Curdione**	**1.3**	**1.6**	**1.3**	**1.6**	**28.7**	**35.6**	**19.8**	**17.7**	**—**	**—**	**—**	**—**
1724	1732	Curcumenol	4.6	3.7	7.1	4.3	—	—	—	—	1.2	0.9	—	0.5
1733	1731	Zerumbone	—	—	—	—	—	0.1	—	—	—	—	—	—
1738	—	Unidentified [a]	1.1	0.7	1.1	1.0	—	—	—	—	0.3	0.2	—	—
1742	—	Unidentified [a]	—	—	—	—	1.8	—	—	—	—	—	—	—
1743	1742	(6*S*,7*R*)-Bisabolone	0.3	0.2	0.4	0.3	—	—	0.2	0.2	2.8	2.3	1.5	1.6
1745	—	Unidentified [a]	2.1	1.6	1.8	1.9	1.4	—	0.6	0.4	—	—	0.2	0.3
1747	1750	Xanthorrhizol	—	—	—	—	—	—	—	—	—	0.2	—	—
1749	—	Unidentified [a]	—	—	—	—	—	1.6	0.6	0.5	—	—	—	—
1752	—	Unidentified [a]	—	—	—	—	—	2.1	—	—	—	—	—	—
1771	1771	*trans*-α-Atlantone	—	—	—	—	—	—	—	—	0.2	0.3	0.5	0.5
1777	1775	Curzerenone A	—	—	—	—	0.2	0.1	—	—	—	—	—	—
1778	**—**	**Unidentified [a]**	**9.0**	**8.7**	**11.0**	**10.3**	**—**	**0.7**	**1.8**	**3.3**	**2.1**	**1.9**	**0.2**	**1.0**
1788	—	Unidentified [a]	1.4	1.6	1.7	1.5	0.3	0.3	1.6	2.3	—	—	0.2	0.1
1827	—	Unidentified [a]	1.6	0.6	1.0	0.9	0.1	0.2	0.6	0.3	1.3	1.6	—	—
1834	**—**	**Curcumenone**	**3.1**	**3.1**	**4.2**	**3.8**	**3.0**	**4.5**	**1.9**	**2.4**	**0.9**	**0.8**	**—**	**0.6**
1836	—	4,5-Epoxygermacrone	2.1	1.0	1.3	1.3	0.3	0.4	1.1	0.7	0.2	0.1	—	—
1929	—	Unidentified [a]	—	—	—	—	—	—	—	—	0.4	0.8	1.3	1.1
1987	—	Zederone	0.4	0.4	0.6	0.6	0.6	0.9	0.3	0.5	—	—	—	—
1992	—	Unidentified [a]	—	—	—	—	—	—	—	—	1.2	2.2	—	3.2

Table 2. *Cont.*

RI_{calc}	RI_{db}	Compound	*Curcuma aromatica* (Green Rhizome)		*Curcuma aromatica* (White Rhizome)		*Curcuma caesia* (Black Rhizome)		*Curcuma zanthorrhiza* (Lime Rhizome)		*Curcuma longa* (Yellow Rhizome)		*Curcuma longa* (Red Rhizome)	
			CA22 (M)	CA22 (D)	CA46 (M)	CA46 (D)	CC38 (M)	CC38 (D)	CZ44 (M)	CZ44 (D)	CL56 (M)	CL56 (D)	CL63 (M)	CL63 (D)
2345	—	(*E*)-Labda-8(17),12-diene-15,16-dial	—	—	—	1.0	—	—	0.2	0.8	—	—	—	—
		Monoterpene hydrocarbons	4.2	8.9	4.1	8.1	0.4	0.5	5.9	5.2	2.1	1.9	3.0	3.4
		Oxygenated monoterpenoids	15.5	10.8	12.7	8.1	2.3	0.6	16.1	7.4	15.1	3.9	3.9	1.7
		Sesquiterpene hydrocarbons	10.3	20.3	10.7	16.0	11.6	7.9	13.8	19.6	27.8	32.1	14.2	15.8
		Oxygenated sesquiterpenoids	52.8	44.8	54.0	49.7	76.1	83.2	58.0	57.7	44.8	50.2	72.4	66.2
		Diterpenoids	0.0	0.0	0.0	1.0	0.0	0.0	0.2	0.8	0.0	0.0	0.0	0.0
		Benzenoid aromatics	0.0	0.0	0.0	0.0	traces	traces	0.0	0.0	traces	traces	traces	traces
		Others	1.0	1.6	0.3	0.6	4.0	0.9	0.5	0.6	0.7	0.4	0.0	0.0
		Total identified	83.8	86.4	81.8	83.4	94.4	93.2	94.5	91.4	90.5	88.6	93.5	87.1

RI_{calc} = Retention index determined with respect to a homologous series of *n*-alkanes on a ZB-5ms column. RI_{db} = Retention index from the databases [25–28]. M = mother (main) rhizome. D = daughter (finger) rhizome. tr = trace (<0.05%). [a] Mass spectra of the unidentified components are presented in Supplementary Figure S2. The major components are highlighted in **bold font**.

Likewise, the lime-colored rhizome essential oils of *C. zanthorrhiza* (CZ44,) were also rich in curzerenone (16.3% and 19.7%) and curdione (19.8% and 17.7%), in addition to germacrone (11.3% and 11.1%) for the mother and daughter rhizomes, respectively. This composition is in marked contrast to that reported for *C. zanthorrhiza* from Thailand with 1,8-cineole (37.6%) and curzerenone (13.7%) as the major components [30]. 1,8-Cineole was in lower concentration (7.2% and 2.5% for the mother and daughter rhizomes) in *C. zanthorrhiza* in this work. A *C. zanthorrhiza* rhizome essential oils from Bogor, Indonesia, on the other hand, was dominated by xanthorrhizol (26.8%), β-curcumene (17.0%), *ar*-curcumene (15.1%), camphor (9.1%), and germacrone (5.4%) [35]. Another sample of *C. zanthorrhiza* from West Java, Indonesia, was composed of β-curcumene (23.4%), *ar*-curcumene (22.1%), curzerene (6.0%), camphor (5.0%), and xanthorrhizol (4.7%) as the dominant constituents [36]. Neither β-curcumene nor xanthorrhizol were detected in the essential oil sample cultivated in North Alabama.

The major components of the rhizome essential oils from *C. longa* CL56 (yellow-colored rhizome) were α-turmerone (12.7% and 14.1%), α-zingiberene (11.4% and 13.9%), *ar*-turmerone (8.3% and 12.6%), and β-sesquiphellandrene (8.9% and 10.0%). The red-colored rhizome variety of *C. longa* (CL63) also yielded essential oils rich in *ar*-turmerone (36.1% and 31.3%), and α-turmerone (15.2% and 13.0%), as well as β-turmerone (=curlone) (15.4% and 13.0%). By comparison, the rhizome essential oils of *C. longa* cultivated in North Alabama, reported previously, showed α-turmerone (13.6–31.5%), *ar*-turmerone (6.8–32.5%), β-turmerone (4.8–18.4%), α-phellandrene (3.7–11.8%), 1,8-cineole (2.6–11.7%), α-zingiberene (0.9–12.5%), and β-sesquiphellandrene (0.7–8.0%) [9]. Two distinct chemical variations were found in the previous examination of *C. longa* cultivated in North Alabama. One group was dominated by turmerones (α-turmerone, *ar*-turmerone, and β-turmerone), while the second group had lower concentrations of turmerones but high concentrations of α-zingiberene and β-phellandrene. Thus, the red-colored rhizome (CL63) belongs to the turmerone-rich chemical group, while the yellow-colored rhizome (CL56) belongs to the second group (high in α-zingiberene).

In order to place the volatile phytochemistry of the *Curcuma* rhizome essential oils in this study into perspective, an agglomerative hierarchical cluster analysis (HCA) was carried out based on the relative concentrations of the major components (Figure 2). There are two clearly defined clusters with at least 50% similarity based on the HCA: Cluster 1 is a cluster made up of CA22 (green rhizome), CA46 (*C. aromatica*, white rhizome) CC38 (*C. caesia*, black rhizome), and CZ44 (*C. zanthorrhiza*, lime rhizome), essential oils and defined by relatively high concentrations of curzerenone (14.7–29.1%), curdione (1.3–35.6%), and germacrone (3.8–14.7%); and Cluster 2, a cluster of CL56 (*C. longa*, yellow rhizome) and CL63 (*C. longa*, red rhizome) rhizome essential oils that were dominated by *ar*-turmerone (8.3–36.1%), α-turmerone (12.7–15.2%), and β-turmerone (5.0–15.4%) (see Table 2).

Interestingly, the volatile phytochemistry of *C. caesia* and *C. zanthorrhiza* rhizomes are very similar (about 90% similarity). Likewise, the green- and white-colored rhizome essential oils of *C. aromatica* are very similar (about 95% similarity). The yellow- and red-colored rhizome essential oils of *C. longa* showed somewhat lower similarity (about 60% similarity).

There are some significant differences in the concentrations of the major components in Cluster 1 (Figure 3). The concentration of curzerenone is significantly greater in *C. caesia* than in either *C. aromatica* or *C. zanthorrhiza*. The concentrations of curdione in *C. aromatica* are significantly lower than those in either *C. caesia* or *C. zanthorrhiza*. Germacrone was significantly lower in *C. caesia* than in either *C. aromatica* or *C. zanthorrhiza*.

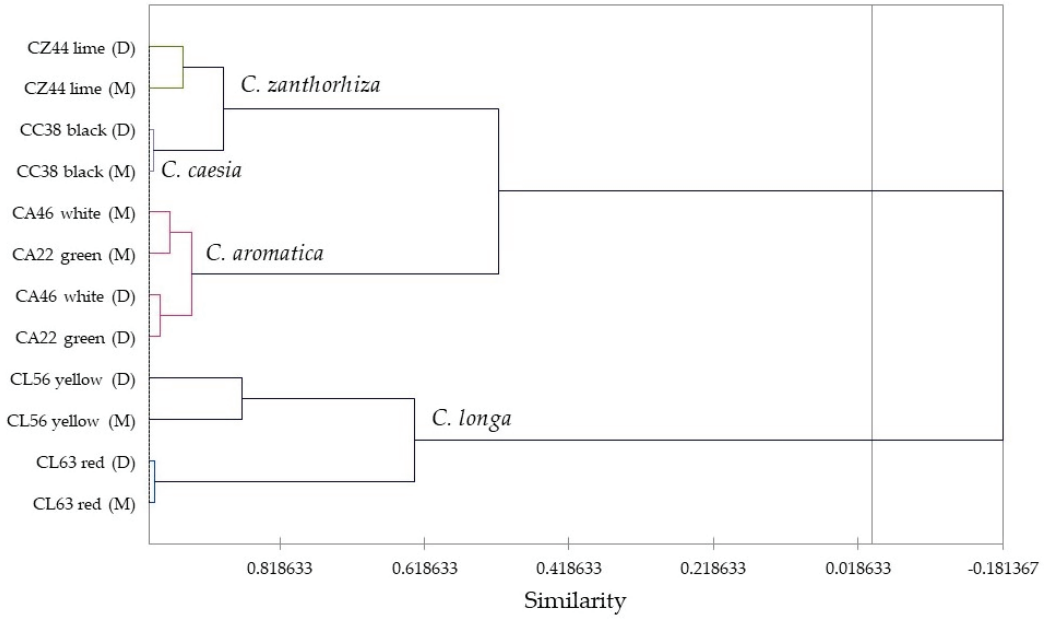

Figure 2. Dendrogram obtained from the agglomerative hierarchical cluster analysis of the rhizome essential oil compositions of Vietnamese *Curcuma* species cultivated in North Alabama.

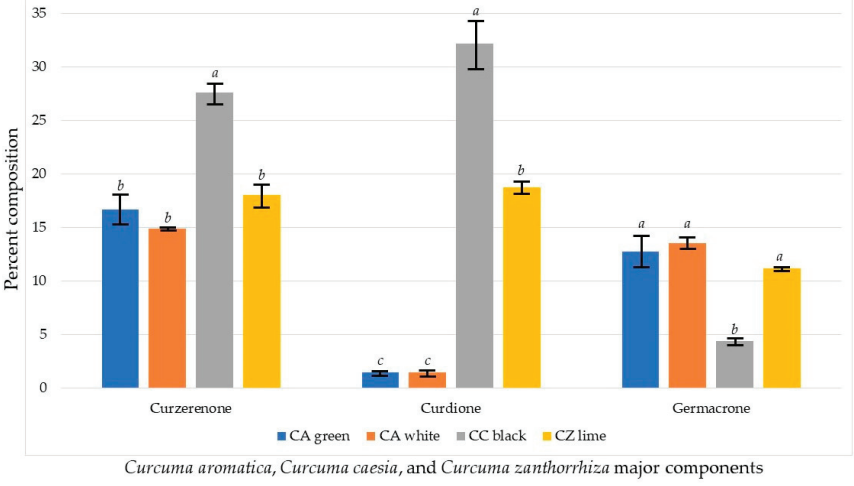

Figure 3. Comparison of the main chemical components of *Curcuma aromatica*, *Curcuma caesia*, and *Curcuma zanthorrhiza*. For each chemical component, bars with the same letter are not significantly different at $p \leq 0.05$.

The significant differences between the essential oils of yellow- and red-colored *C. longa* are the concentrations of *ar*-turmerone (much higher in the red rhizome variety) and β-turmerone (also higher in the red rhizome variety). The concentrations of α-turmerone in the red and yellow varieties are not significantly different (Figure 4). Nevertheless, although the compositions of yellow- and red-colored rhizomes of *C. longa* are notably different (60% similarity), they are comparable to the respective chemical profiles of *C. longa* from tropical Asian collections [3].

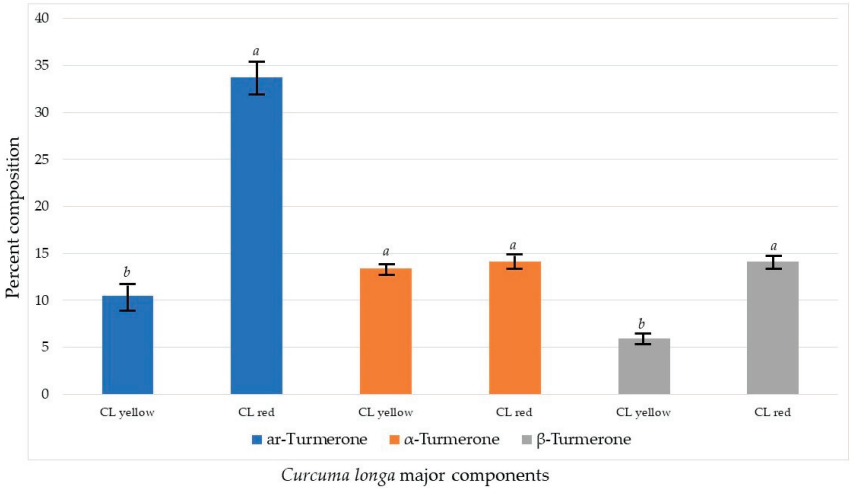

Figure 4. Comparison of the main chemical components of *Curcuma longa*. For each chemical component, bars with the same letter are not significantly different at $p \leq 0.05$.

3.2. Enantiomeric Distribution of Terpenoids in Curcuma Essential Oils

The enantiomeric distributions of terpenoid components in *Curcuma* rhizome essential oils have been determined by enantioselective GC-MS (Table 3). Although only found in trace quantities, when detected by chiral GC-MS (*C. aromatica*, *C. caesia*), the (−)-α-thujene predominated. (−)-α-Pinene was the dominant enantiomer in all samples. (−)-β-Pinene also predominated in all samples, but was especially dominant in the essential oils of *C. zanthorrhiza* (CZ44) and *C. longa* (CL56 and CL 63). (+)-Camphene was the dominant enantiomer in *C. aromatica*, *C. caesia*, and *C. zanthorrhiza*.

α-Phellandrene and δ-3-carene, only detected in the essential oils of *C. longa*, were both exclusively the (+) enantiomers. (−)-Limonene was the major stereoisomer in the *Curcuma* essential oils, but was nearly racemic in *C. zanthorrhiza*. (−)-Sabinene predominated is all *Curcuma* rhizome essential oils where it was detected. (−)-Linalool was the major enantiomer in nearly all *Curcuma* samples, but was particularly abundant in *C. caesia* (CC38), *C. zanthorrhiza* (CZ44), and the yellow-rhizome *C. longa* (CL56). Interestingly, however, the red-rhizome *C. longa* (CL63) exhibited (+)-linalool as the major enantiomer. Likewise, (−)-α-terpineol was the dominant enantiomer in all *Curcuma* essential oils. Camphor was not found in *C. longa*, but (+)-camphor was the dominant enantiomer in *C. aromatica*, *C. caesia*, and *C. zanthorrhiza*. The major enantiomer of terpinen-4-ol in *Curcuma* essential oils was (−)-terpinen-4-ol, although the distribution was nearly racemic in *C. zanthorrhiza*.

δ-Elemene was nearly racemic in all of the *Curcuma* essential oils, whereas *trans*-β-elemene was exclusively the (−) enantiomer. Both (*E*)-β-caryophyllene and δ-cadinene were 100% (−) enantiomers, while germacrene D and β-bisabolene were exclusively the dextrorotatory stereoisomers.

Table 3. Enantiomeric distribution of terpenoid components in Vietnamese *Curcuma* rhizome essential oils cultivated in North Alabama.

Compound	*Curcuma aromatica* (CA22, Green Rhizome)				*Curcuma aromatica* (CA46, White Rhizome)				*Curcuma caesia* (CC38, Black Rhizome)			
	Mother		Daughter		Mother		Daughter		Mother		Daughter	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
α-Thujene	14.1	85.9	12.5	87.5	15.9	84.1	—		18.6	81.4	—	
α-Pinene	23.5	76.5	26.1	73.9	21.7	78.3	24.5	75.5	—		22.2	77.8
Camphene	85.0	15.0	81.3	18.7	83.4	16.6	82.5	17.5	—		—	
Sabinene	28.3	71.7	36.6	63.4	33.7	66.3	37.0	63.0	17.8	82.2	46.3	53.7
β-Pinene	47.8	52.2	43.3	56.7	41.3	58.7	40.0	60.0	43.5	56.5	38.5	61.5
α-Phellandrene	—		—		—		—		—		—	
δ-3-Carene	—		—		—		—		—		—	
Limonene	19.2	80.8	32.2	67.8	19.7	80.3	33.8	66.2	14.9	85.1	29.9	70.1
Linalool	39.7	60.3	20.3	79.7	48.5	51.5	29.2	70.8	0.0	100.0	0.0	100.0
Camphor	99.1	0.9	99.2	0.8	99.1	0.9	99.3	0.7	100.0	0.0	100.0	0.0
Terpinen-4-ol	30.7	69.3	37.0	63.0	29.9	70.1	39.4	60.6	25.1	74.9	33.2	66.8
δ-Elemene	48.4	51.6	48.1	51.9	47.3	52.7	48.1	51.9	49.1	50.9	48.6	51.4
α-Terpineol	20.6	79.4	31.7	68.3	28.5	71.5	20.7	79.3	16.3	83.7	15.8	84.2
Carvone	23.5	76.5	—		25.5	74.5	—		—		—	
trans-β-Elemene	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
(*E*)-β-Caryophyllene	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
Germacrene D	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
β-Bisabolene	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	—		—	
δ-Cadinene	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	—		0.0	100.0
(*E*)-Nerolidol	—		—		—		—		—		—	

Compound	*Curcuma zanthorrhiza* (CZ44, lime rhizome)				*Curcuma longa* (CL56, yellow rhizome)				*Curcuma longa* (CL63, red rhizome)			
	Mother		Daughter		Mother		Daughter		Mother		Daughter	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
α-Thujene	—		—		—		—		—		—	
α-Pinene	21.6	78.4	11.7	88.3	11.9	88.1	11.4	88.6	34.7	65.3	33.8	66.2
Camphene	91.6	8.4	90.1	9.9	—		—		—		—	
Sabinene	—		—		20.6	79.4	19.5	80.5	—		—	
β-Pinene	2.8	97.2	2.5	97.5	12.3	87.7	11.2	88.8	24.2	75.8	11.9	88.1
α-Phellandrene	—		—		100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
δ-3-Carene	—		—		100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
Limonene	41.3	58.7	42.7	57.3	12.5	87.5	12.2	87.8	22.9	77.1	33.8	66.2
Linalool	10.9	89.1	9.0	91.0	17.9	82.1	0.0	100.0	82.0	18.0	—	

Table 3. *Cont.*

Compound	*Curcuma zanthorrhiza* (CZ44, lime rhizome)				*Curcuma longa* (CL56, yellow rhizome)				*Curcuma longa* (CL63, red rhizome)			
	Mother		Daughter		Mother		Daughter		Mother		Daughter	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
Camphor	99.6	0.4	99.7	0.3	—		—		—		—	
Terpinen-4-ol	42.6	57.4	45.7	54.3	11.0	89.0	26.0	74.0	26.4	73.6	22.5	77.5
δ-Elemene	45.9	54.1	47.6	52.4	44.3	55.7	48.8	51.2	—		—	
α-Terpineol	17.3	82.7	15.3	84.7	11.0	89.0	10.6	89.4	41.5	58.5	13.1	86.9
Carvone	—		—		—		—		—		—	
trans-β-Elemene	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	—		0.0	100.0
(*E*)-β-Caryophyllene	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
Germacrene D	100.0	0.0	98.8	1.2	100.0	0.0	100.0	0.0	—		—	
β-Bisabolene	100.0	0.0	100.0	0.0	91.9	8.1	92.0	8.0	92.6	7.4	91.6	8.4
δ-Cadinene	0.0	100.0	0.0	100.0	—		—		—		—	
(*E*)-Nerolidol	—		—		—		—		0.0	100.0	0.0	100.0

(+) = dextrorotatory enantiomer, (−) = levorotatory enantiomer.

4. Conclusions

The rhizome essential oils of *C. aromatica*, *C. caesia*, *C. longa*, and *C. zanthorrhiza* that were cultivated in North Alabama showed wide variation in composition compared to essential oils from other geographical locations. Nevertheless, the essential oil yields and composition provide evidence that *Curcuma* can be successfully cultivated in North Alabama and may provide additional sources of these species for both culinary and herbal medicinal uses. The knowledge of their relative oil yields and composition could help in value-addition for either fresh rhizomes or dry herbal markets. The four species showed specific essential oil components, which are known to have extensive pharmacological activity separately or in combination with curcuminoids. The species can be used to tailor herbal medicines to combat particular ailments. The cultivation of specific varieties to cater to niche markets could not only benefit the farmers, but also have an impact on the socio-economic sustainability of rural Alabama in particular and the southeastern U.S. in general. As far as we are aware, this is the first report of the essential oil compositions, including enantiomeric distributions for these *Curcuma* species cultivated in North America. Among the four species, the *C. longa* species that combine high yield with high curcumin have been found to be suitable for cultivation. However, the remaining species have economic potential, for example *C. zanthorrhiza* is well known for its antimicrobial activity against common human pathogens to cater to herbal companies interested in varieties that are high in a certain essential oil component.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050360/s1, Figure S1: Gas chromatograms of rhizome essential oils of *Curcuma* varieties cultivated in North Alabama; Figure S2: Mass spectra of unidentified components of *Curcuma* rhizome essential oils.

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Essential Oils from Leaves of *Juniperus thurifera* L., Exhibiting Antioxidant, Antifungal and Antibacterial Activities against Antibiotic-Resistant Microbes

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Abstract: The antioxidant, antibacterial and antifungal properties of essential oils (EOs) of *Juniperus thurifera* L., a plant utilized in traditional, herbal medicine, were investigated. The EOs were extracted by use of a Clevenger apparatus and phytochemicals identified by gas chromatography coupled with mass spectrometry (GC/MS/MS). The antioxidant capacity of EOs of *J. thurifera* was determined by 2,2-diphenyl-1-picrylhydrazil (DPPH), total antioxidant capacity (TAC), and ferric reducing antioxidant power (FRAP). Antimicrobial activity of EOs of *J. thurifera* was determined against four fungal strains, *Candida albicans*; ATCC 10231, *Aspergillus niger*; MTCC 282, *Aspergillus flavus*; MTCC 9606 and *Fusarium oxysporum*; MTCC 9913 and four bacterial strains, *Staphylococcus aureus*; ATCC 6633, *Escherichia coli*; K12, *Bacillus subtilis*; DSM 6333, and *Pseudomonas aeruginosa*; CIP A22, by use of the disk diffusion method, and microdilution method used to determine the minimum inhibitory concentration (MIC). EOs of *J. thurifera* consisted of 31 compounds and were dominated by α-thujene (25%), elemol (12%) and muurolol (12%). Antioxidant activity recorded an IC_{50} of 24 ± 0.71 µg/mL (DPPF), EC50 of 0.19 ± 0.01 mg/mL (FRAP), and $9.3 \times 10^2 \pm 38$ mg EAA/g (TAC). The EOs of *J. thurifera* exhibited significant antibacterial activity against all bacterial strains under investigation, especially *P. aeruginosa*; CIP A22 with an inhibition diameter of 28 ± 1.5 mm and MIC of $4.8 \times 10^{-2} \pm 0.001$ µg/mL. EOs of *J. thurifera* also exhibited significant antifungal activity against *C. albicans*; ATCC 10231 and *F. oxysporum*; MTCC 9913 with an activity of 21 ± 2.1 mm, 32 ± 2.3%, and MIC of $9.5 \times 10^{-2} \pm 0.001$ Bioactive molecules found in EOs of *J. thurifera* could be used as an alternative solution to antibiotics available on the market to combat microbial resistance.

Keywords: juniper; natural products; tree; leaves; disease; bioactive compounds; microbial resistance

1. Introduction

Thuriferous juniper (*Juniperus thurifera* L.) is a monoecious, dioecious, conifer tree or shrub with scaly leaves of the cypress family (Cupressaceae) [1]. It plays an important role in the western Mediterranean basin and is regarded as a keystone species of low-temperature-adapted open woodlands, with steppe-like undercover [2]. In Morocco, the area of this species, which constitutes the upper limit of the forest in the Atlas Mountains, has been considerably restricted and, the vast majority of the stands have been degraded by over-exploitation and over-grazing, aggravated by an almost total absence of regeneration. Its current surface area, in the Atlas Mountains of Morocco, is estimated to be 20,000 ha [3,4]. Thuriferous juniper, with its extraordinary resistance, remarkable ability to withstand very severe climatic conditions, and indifference to the physical nature of soils, longevity of as much as 500 years, is unquestionably the predominant tree of the high Moroccan mountains [5]. Development of these natural plant resources is mainly based on extraction of essential oils (EOs), which are high value-added products, employed in the pharmaceutical, cosmetics and food industries [6–8]. The genus *Juniperus* contains essential medicinal plants with a long history of usage in traditional medicine. Its leaves are used to treat diabetes, diarrhoea, and rheumatism as a decoction [9]. Leaves and berries of *Juniperus* are utilized as an oral hypoglycemic medication [10], while leaves are used to treat bronchitis and as a diuretic [9]. Studies of the biological and biotechnological activities of the phytochemical compounds of plants is of interest and the antimicrobial activities of EOs have been reported [6,11–13]. These activities are attributed to oxygenated mono-terpenes [6]. Use of natural molecules to inhibit oxidation of fat, its consequences on health and its economic repercussions have been the subject of several studies [6,14]. Results of several studies of the antioxidant activities of EOs from a variety of aromatic plants have demonstrated that these properties are mainly ascribed to the presence of compounds containing hydroxyl group(s) [15–17]. Recently, the EOs and aqueous extracts of plants have attracted interest because of their richness in natural biologically active constituents including antioxidant, antimicrobial and insecticidal properties.

Under the existing restricted and inadequate arsenal of new therapies, the list of microorganisms that are becoming resistant to all commonly used antibiotics is growing, prompting the discovery of alternative classes of medications to prevent significant public health concerns, unconventional therapeutic interventions derived from natural resource exploitation have been intensively investigated [18,19]. Objectives of this study were to describe the chemical composition of the EOs of leaves of *J. thurifera* collected from the Jbel lakraa Massif in the Eastern, Middle Atlas of Morocco and to compare the results to those of previous studies and investigate the antioxidant, antibacterial and antifungal activities of the EOs, so that an evaluation of the economic value of the EOs of *J. thurifera* and their potential as replacements for antibiotics available on the market to combat microbial resistance could be conducted.

2. Materials and Methods

2.1. Extraction of EOs from J. thurifera

J. thurifera was harvested from the mountains (lat: 33.68093368; long: 4.30823143) during October 2021, which was autumn. Specimens were identified by a botanist in the department of biology, Faculty of Sciences-FSDM-USMBA-Fez, and the plant is deposited in the Herbarium under number (FJT/02D20). Leaves were cleaned and subsequently dried at 35 °C for 72 h in a ventilated oven. Dried leaves were crushed with an electric blender, then EOs extracted by hydro-distillation on a Clevenger-type extractor [15]. Briefly, 200 g of the ground leaf material was mixed with 750 mL distilled water (dH_2O) and extracted for about 120 min. Samples were partitioned into hexane (10%). At least three replicates were performed in this study.

2.2. GC/MS/MS Analysis of EOs

Constituents of EOs were identified and quantified by use of gas chromatography (TQ8040 NX; Shimadzu, Tokyo, Japan) attached to a triple quadrupole, tandem mass spectrometer (GC-MS). Chromatography was conducted on an apolar, capillary column RTxi-5 Sil MS column (30 m × 0.25 mm ID × 0.25 µm). Helium was used as carrier gas and the injection volume was 1 µL. Temperatures of the source and the interface were 200 °C and 280 °C, respectively. The chromatographic system was programmed with splitless injection (split opening at 4 min), injection temperature of 250 °C and pressure of 37.1 kPa. Temperature was programmed with an initial temperature of 50 °C for 2 min, ramp 1 was 5 °C/min to 160 °C for 2 min and ramp 2 was 5 °C/min to 280 °C for 2 min. Identification of phytochemicals in EOs was conducted by comparing the obtained retention indices with those of chemical compounds in the literature database [20].

2.3. Antioxidant Activity

2.3.1. DPPH Test

Antioxidant activity was determined by use of the DPPH assay according to previously published method [21]. Briefly, 800 µL of a methanolic solution of DPPH (0.2 mM) was mixed with 200 µL of different dilutions of EOs of *J. thurifera* (0–1 mg/mL), and subsequently incubated in the dark at RT for 30 min. Absorbances of samples were recorded at 517 nm and compared to those of a control consisting of 800 µL of DPPH solution. Samples, positive controls, quercetin or BHT were prepared under the same operating conditions. Decay of absorbance was measured with a spectrophotometer and percent inhibition (I%) calculated (Equation (1)).

$$I\ (\%) = [(T0 - Tx)/T0] * 100 \quad (1)$$

By performing kinetics of this activity, concentrations corresponding to 50% inhibition (IC_{50}), expressed as µg/mL, were determined, where the least IC_{50} corresponds to the greatest efficiency of EOs.

2.3.2. TAC Test

Antioxidant activity was determined by placing 100 µL of EOs at various concentrations after adding 1000 µL of a reagent composed of 0.6 M H_2SO_4, 28 mM Na_2PO_4 and 4 mM $(NH_4)_2MoS_4$. Then, the tubes were tightly closed and incubated at 95 °C for 90 min. After cooling, absorbances were measured at 695 nm. The negative control consisted of 100 µL of methanol after the addition of 1000 µL of the above reagent [22]. The samples and controls were incubated under the same conditions. The obtained results were represented in mg ascorbic acid equivalents per gram (mg EAA/g).

2.3.3. FRAP Test

Reducing power was recorded by placing 200 µL of sample at several concentrations, into 500 µL of 0.2 M phosphate buffer (pH = 6.6), followed by 500 µL of 1% $K_3Fe(CN)_6$ in dH_2O. Mixtures were subsequently placed into a water bath and incubated at 50 °C for 20 min. Next, about 500 µL trichloroacetic acid (TCA, 10%) was added followed by centrifugation. A 500 µL aliquot of the supernatant was transferred to another tube followed by the addition of 500 µL of dH_2O and 100 µL of freshly-prepared $FeCl_3$ (1%) in dH_2O. Similarly, a blank without sample was included by replacing EOs of *J. thurifera* with methanol. Absorbances of reaction media were recorded at 700 nm and compared to the methanol blank, which allowed calibration of the apparatus (UV-VIS spectrophotometer). Positive controls were a solution of the standard antioxidants BHT and quercetin [23].

2.4. Antimicrobial Activity of EOs of J. thurifera

2.4.1. Microbial Strains Tested

Antimicrobial activity of *J. thurifera* EOs against four fungal strains, *Candida albicans*, ATCC 10231; *Aspergillus niger*, MTCC 282; *Aspergillus flavus*, MTCC 9606 and *Fusarium*

oxysporum, MTCC 9913 and four strains of bacteria, *Staphylococcus aureus*, ATCC 6633; *Escherichia coli*, K12; *Bacillus subtilis*, DSM 6333 and *Pseudomonas aeruginosa*, CIP A22. The fungal and bacteria strains were provided by Sidi Mohammed Ben Abdellah University (Fez, Morocco) and Hassan II University Hospital (Fez, Morocco), respectively.

2.4.2. Assessment of Antimicrobial Activity

The antimicrobial activity of *J. thurifera* EOs was determined by use of the disc diffusion method [24]. Petri dishes containing Mueller–Hinton (MH) and Malt Extract (ME) culture media were inoculated with the four bacterial strains and *C. albicans*, respectively, by the double-layer method, from cultures freshly grown in MH and ME medium, decimal dilutions were made in sterile saline (0.9%) until turbidity of 0.5 McFarland (10^8 CFU/mL) was reached, 100 µL were added to tubes containing 5 mL of soft agar (0.5% agar), then the inoculated tubes were spread in Petri dishes containing MH and ME medium. For *A. niger*, *A. flavus*, and *F. oxysporum* the antifungal activity was determined by the direct confrontation method in the ME medium. Sterile 6 mm Whatman paper discs were positioned into the centre of the petri dish and then impregnated with 20 µL of *J. thurifera* EOs, and also with conventional antimicrobial drugs; streptomycin and erythromycin for bacterial strains and fluconazole for fungal strains according to the methodology of the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Then, bacteria- and fungi-inoculated dishes were incubated at temperatures of 30 °C and 37 °C optimal for the bacterial and fungal strains and *C. albicans*, respectively. Inhibition diameters and percentages of inhibition were calculated 18–24 h post inoculation (hpi) for the bacterial strains and after 24–48 hpi for *C. albicans*, and 7 days post inoculation for *F. oxysporum*, *A. niger* and *A. flavus* [24,25].

2.4.3. Minimum Inhibitory Concentration (MIC) Determination

Minimum inhibitory concentrations (MIC) of *J. thurifera* EOs against the four bacterial and four fungal strains were determined by use of the microdilution as previously described [25]. Briefly, a sterile 96-well microplate was used and 50 µL of sterile MH or ME medium was added for bacterial and fungal strains, respectively. Serially diluted EOs of *J. thurifera* at a volume of 100 µL prepared in 10% (*v*/*v*) DMSO was pipetted into the first row. This was followed by the addition of 30 µL of microbial strains. Plates were incubated for 24 h, 48 h or 7 d for bacteria, *C. albicans* and fungi (*Fusarium oxysporum*, *A. niger*, *A. flavus)*, respectively; at 37 °C or 30 °C [25,26]. Each well received 20 µL of water 2,3,5-triphenyl tetrazolium chloride solution (0.2%) to visualize bacterial growth. MIC was defined as the least concentration that did not create a red colour [26].

2.5. Statistical Analyses

Results were expressed as means of triplicates ± SD (standard deviation). Shapiro–Wilks test was employed to determine the normality of distribution, while the *t*-test was used to check for homogeneity of variances. Analysis of variance (ANOVA) was performed, with Tukey's HSD test, as a post hoc test for multiple comparisons. Differences were considered significant at probability level (p) < 0.05.

3. Results and Discussion

3.1. Identification of Chemicals Comprising EOs of J. thurifera by GC/MS

The yield of EOs of 0.96%, from leaves of *J. thurifera* provided was greater than that reported previously [27]. Essentially, all of the mass of the EOs of *J. thurifera* (99.99%) was accounted for by 31 phytochemical compounds (Table 1 and Figure 1). Previously, 99.46% of the mass of EOs of *J. thurifera* was reported to be accounted for by 24 compounds [28]. The phytochemical composition of the EOs of *J. thurifera* is dominated by α-thujene (25%), elemol (12%) and muurolol (12%) (Figure 2). The chemical composition of EOs of *J. thurifera* was quantitatively and qualitatively different from that reported previously [27]. In another recent study β-pinene (36%) were determined to be the predominant compounds in EOs of *J. thurifera*, whereas in this study β-pinene accounted for only 1.9% of the mass of EOs.

Table 1. Phytochemical compounds identified by GC/MS/MS in EOs of *J. thurifera*.

Peak	Retention Time	Name	KI		Area (%)
			Calculated	Literature	
1	7.666	Sabinene	975	969	0.95
2	7.886	α-Pinene	939	948	3.09
3	9.031	α-Thujene	930	897	24.98
4	9.487	β-Pinene	979	972	1.94
5	10.096	3-Carene	1011	948	3.39
6	10.554	o-Cymene	1024	1042	4.45
7	10.707	Limonene	1029	1018	0.37
8	11.583	γ-Terpinene	1059	998	0.71
9	11.927	Sabina ketone	1120	1041	1.10
10	12.416	Terpinolene	1088	1052	0.52
11	12.798	Linalool	1090	1082	3.66
12	12.855	Thujanol	1138	1041	1.05
13	13.565	Dihydro carveol	1193	1109	0.44
14	15.258	Limonen-10-ol	1289	1137	12.39
15	15.647	α-Terpineol	1017	1143	0.92
16	17.170	Linalool acetate	1257	1272	1.97
17	18.925	Decadienal	1293	1274	0.38
18	19.176	p-Methylacetophenone	1446	1113	0.42
19	19.865	β-Terpineol	1144	1333	1.04
20	23.828	α-Muurolene	1500	1440	0.66
21	24.199	Selinene	1498	1435	1.02
22	24.317	γ-Cadinene	1513	1469	2.79
23	25.106	Elemol	1549	1522	12.20
24	26.748	β-Oplopenone	1607	1540	0.85
25	27.565	α-Eudesmol	1607	1626	2.43
26	27.830	Cadinol	1640	1580	1.81
27	27.886	Muurolol	1642	1580	11.58
29	28.403	Ethyl pentanoat	901	1068	0.52
30	31.385	Geranyl propanoate	1477	1536	1.32
31	32.545	Cymene	1426	1569	1.04
		Total			99.99%

3.2. Antioxidant Activity

When antioxidant activities of EOs of *J. thurifera* were evaluated by three methods (Figure 3a), the percentage of inhibition of free radical (DPPH) was directly proportional to the concentrations of the EOs of *J. thurifera*. For a concentration of 13 µg/mL of EOs of *J. thurifera*, the percentage of DPPH inhibition was 85 ± 0.24% and for a concentration of 27 µg/mL the percentage of inhibition was approximately 91 ± 0.17% (Figure 3a). Antioxidant capacity was determined from the IC_{50}, which is the concentration necessary to reduce 50% of the DPPH radical. The smaller the IC_{50} value, the greater the antioxidant activity of a compound [29]. Free radical activities of EOs of *J. thurifera*, BHT and quercetin revealed that IC_{50} of EOs of *J. thurifera* is of the order of 23.6 ± 0.71 µg/mL,

14.2 ± 0.14 µg/mL and 15.9 ± 0.56 µg/mL (Figure 3b), respectively. Evaluation of antioxidant capacity by use of the FRAP method revealed that the effective concentration (EC-50) is in the range of 0.19 ± 0.01 mg/mL (EOs of *J. thurifera*), 3.6 × 10^{-2} ± 0.003 mg/mL (BHT) and 2.8 × 10^{-2} ± 0.002 mg/mL (quercetin) (Figure 4a). Total antioxidant capacity (Figure 4b) of EOs of *J. thurifera* was 9.3 × 10^2 ± 38 mg EAA/g versus 5.3 × 10^2 ± 22 mg EAA/g (BHT) and 6.6 × 10^2 ± 46.67 mg EAA/g (quercetin).

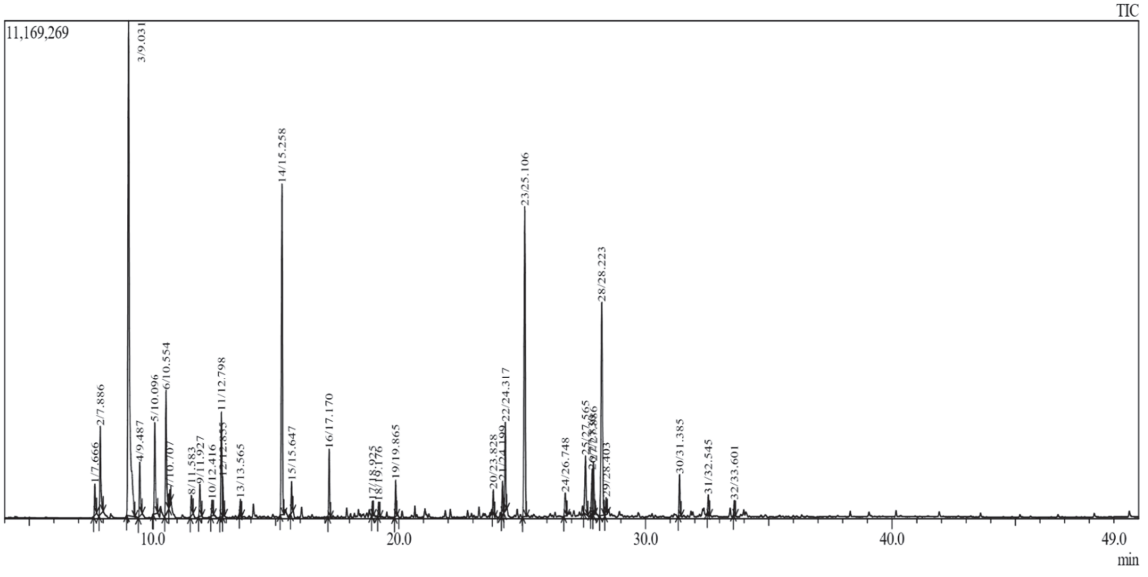

Figure 1. Chromatograph of compounds identified by GC/MS in EOs of *J. thurifera*. Peaks represent absolute abundances, whereas numbers on the x-axis represent retention times in min.

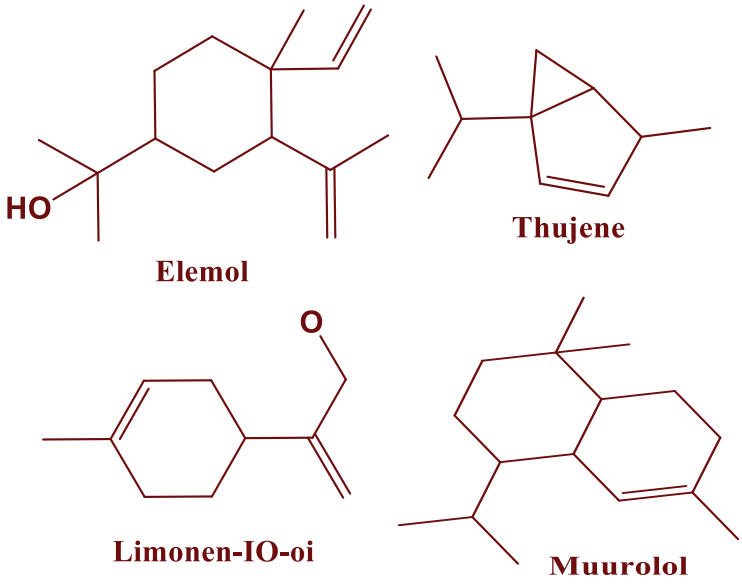

Figure 2. Molecular structures of phytochemical compounds in EOs of *J. thurifera*.

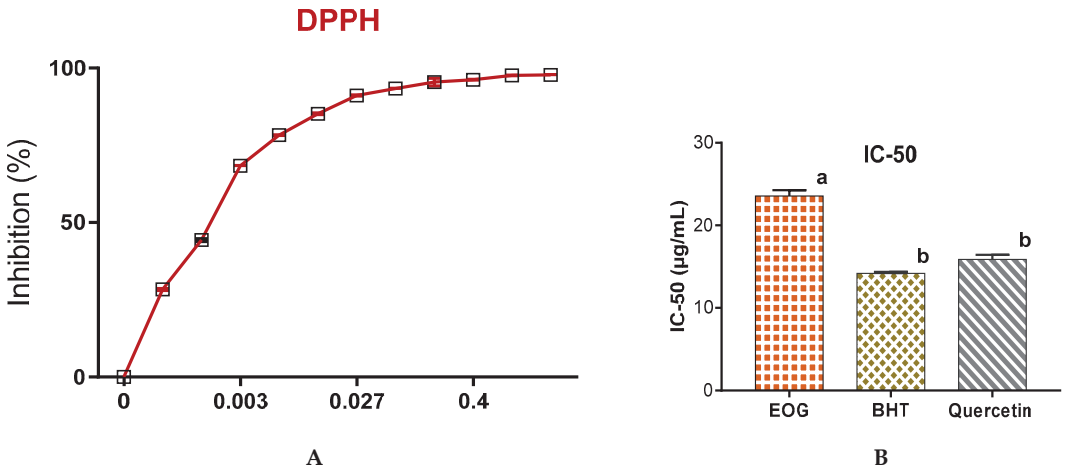

Figure 3. Antioxidant activities of EOs of *J. thurifera* by the DPPH method (**A**) and the concentration of IC-50 (**B**). Bars with the same letters do not differ significantly ($p < 0.05$).

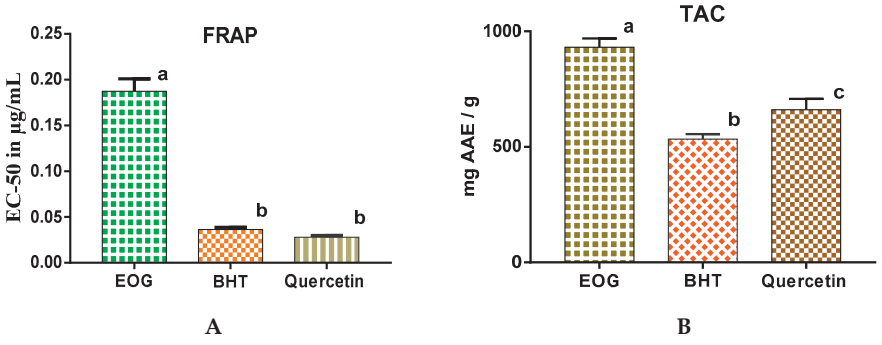

Figure 4. Antioxidant activities of EOs of *J. thurifera* by the FRAP method (**A**) and antioxidant capacity of EOs of *J. thurifera* (**B**). Bars with the same letters do not differ significantly ($p < 0.05$).

Assessment of oxidative stress (OS, oxidation in vivo) has become important since this type of oxidation can be involved in several health effects including rheumatoid arthritis, atherosclerosis, diabetes, aging and cancer [30–32]. Natural antioxidants present in plant extracts and EOs can provide protection against OS by two main mechanisms, namely scavenging reactive oxygen species (ROS) and blocking lipid peroxidation [33,34]. There is a correlation between the antioxidant power of EOs and its phytochemical composition. In this context, it has been previously documented that the antioxidant capacities of EOs are associated with their phytochemical composition via the hydroxyl function present in their constituents, and the richer an oil is in phenolic compounds and terpenes, the more effective its antioxidant capacity [35–40].

The phytochemical profile of EOs of *J. thurifera* (Table 1) revealed that EOs of *J. thurifera* are rich in terpenic compounds, such as thujene, γ-terpinene, cymene and linalool which are known for their antioxidant potentials [41]. Recent studies have shown that sabinene is an antioxidant compound [42]. Similarly, cymene possesses a potent anti-nociceptive behavior although it exhibited lesser antioxidant potential [43].

3.3. Antimicrobial Activity of J. thurifera EOs

3.3.1. Antibacterial Activity of *J. thurifera* EOs

EOs extracted from leaves of *J. thurifera* exhibited antibacterial activity in comparison with the concentration used and which antibiotic was used, streptomycin sulphate or erythromycin especially against *P. aeruginosa* CIP A22 with an inhibition diameter of 27.67 ± 1.53 mm and a MIC of 0.0475 ± 0.00 µg/mL, against *S. aureus*, ATCC 6633 with an inhibition diameter of 20.33 ± 0.58 mm and a MIC of 0.095 ± 0.00 µg/mL, against *E. coli* K12 with an inhibition diameter of 15.67 ± 3.05 mm and a MIC of 0.095 ± 0.00 µg/mL and against *B. subtilis* DSM 6333 with an inhibition diameter of 14.33 ± 1.15 mm and a MIC of 0.095 ± 0.00 µg/mL (Table 2).

Table 2. Antibacterial activity of *J. thurifera* EOs in comparison with the antibiotics streptomycin and erythromycin.

		***Staphylococcus aureus* ATCC 6633**	***Escherichia coli* K12**	***Bacillus subtilis* DSM 6333**	***Pseudomonas aeruginosa* CIP A22**
EOs of *J. thurifera*	Antibacterial activity (mm)	20.33 ± 0.58 [a]	15.67 ± 3.05 [b]	14.33 ± 1.15 [b]	27.67 ± 1.53 [c]
	MIC (µg/mL)	0.095 ± 0.00 [a]	0.095 ± 0.00 [a]	0.095 ± 0.00 [a]	0.0475 ± 0.00 [b]
Streptomycin	Antibacterial activity (mm)	11 ± 1.00	Rs	Rs	Rs
	MIC (µg/mL)	1.56 ± 0.00	-	-	-
Erythromycin	Antibacterial activity (mm)	Rs	Rs	Rs	Rs
	MIC (µg/mL)	-	-	-	-
DMSO 10%	Antibacterial activity (mm)	Rs	Rs	Rs	Rs

Mean values (±SD, n = 3) followed by different letters in the same row are significantly different according to a mean analysis (Student t-test) and an analysis of variance (One-way ANOVA; Tukey's test, $p \leq 0.05$). MIC: minimum inhibitory concentration; Rs: resistance.

Antibacterial activity of *J. thurifera* EOs might be due to their chemical composition, *J. thurifera* EOs are rich in terpene compounds, especially thujene, γ-terpinene, cymene, and linalool which are well known for their antibacterial activity [41]. They are also rich with sabinene and cymene which are compounds with antibacterial activity [44]. Results of the study reported here were different from results of a previous study [44], which indicated that extracts of *J. thurifera* L. leaves growing in eastern Algeria were active only against *S. aureus*, ATCC and methicillin-resistant *S. aureus* bacteria and the greatest activity with an inhibition diameter of 14 mm for a concentration of 1 g/mL. However, no inhibition was detected for extracts against *E. coli* ATCC or *P. aeruginosa* ATCC. However, the antibacterial activity of extracts of leaves of *Juniperus phoenicea* L was observed against both Gram-positive and Gram-negative bacteria [45]. *J. thurifera* EOs exhibited significant antibacterial activity against Gram-positive and Gram-negative bacteria, especially against *S. aureus*, *E. coli*, and *P. aeruginosa* with inhibition diameters of 31.12 ± 3.11, 13.23 ± 2.59, and 18.27 ± 2.29 mm, respectively [46]. Those results were similar to those observed in the study, the results of which are presented here for *E. coli*, but are the opposite of the results for *S. aureus* and *P. aeruginosa*, for which the greater antibacterial activity might have been due to the different physicochemical composition of the EOs observed in the study reported here, which are consistent with results of several other studies [47], which found *S. aureus* was sensitive to the EOs of *J. thurifera* from Algeria. Furthermore, two strains of *Pseudomonas* proved to be resistant, [28]. In that study, the EOs from twigs of *J. thurifera* collected in the Eastern range of the Middle Atlas Mountains of Morocco exhibited significant antibacterial activity against *E. coli*, *B. subtilus*, *M. luteus*, and *S. aureus.* Similarly, EOs of *J. thurifera* had significant antibacterial activity against *S. aureus*, ATCC 33862 with an inhibition diameter of 27 mm and MIC of 450 µL/mL, against *E. coli*, ATCC 25922 with an inhibition diameter

of 25.6 mm and MIC of 530 µL/mL and against *P. mirabilis*, ATCC 7002 with an inhibition diameter of 18.8 mm and MIC of 930 µL/mL [48].

3.3.2. Antifungal Activity of *J. thurifera* EOs

When compared with the fungicide fluconazole in the in vitro evaluation of antifungal activity of *J. thurifera* EOs against *A. niger*, *A. flavus*, *F. oxysporum*, and *C. albicans* in the disc diffusion test, these EOs exhibited significant activity against *F. oxysporum*, MTCC 9913 with percent inhibition of 32.47 ± 2.25 and MIC values of 0.095 µg/mL as well as with an inhibition diameter of 21.33 ± 2.08 mm and a MIC value of 0.095 µg/mL against *C. albicans*; ATCC 10231 (Table 3), In addition, *J. thurifera* EOs exhibited antifungal activities against *F. oxysporum* and *C. albicans*. However, *J. thurifera* EOs did not exhibit antifungal activity against *A. niger* or *A. flavus*. The antifungal activity of *J. thurifera* EOs may be mainly due to their chemical composition, *J. thurifera* EOs are particularly rich in thujene, pinene, and limonene which are well known for their antimicrobial activity, especially antifungal activity [16,17].

Table 3. Antifungal activity of *J. thurifera* EOs in comparison with fluconazole.

		Candida albicans **ATCC 10231**	***Aspergillus niger*** **MTCC 282**	***Aspergillus flavus*** **MTCC 9606**	***Fusarium oxysporum*** **MTCC 9913**
EOs of *J. thurifera*	Antifungal activity	21.33 ± 2.08 mm [a]	0.00 ± 0.00% [b]	0.00 ± 0.00% [b]	32.47 ± 2.25% [c]
	MIC (µg/mL)	0.095 ± 0.00 [a]	-	-	0.095 ± 0.00 [a]
Fluconazole	Antifungal activity	Rs	8.20 ± 2.02% [a]	Rs	30.77 ± 0.58% [b]
	MIC (µg/mL)	-	7.125 ± 0.00 [a]	-	3.125 ± 0.00 [b]
DMSO 10%	Antifungal activity	Rs	Rs	Rs	Rs

Mean values (±SD, n = 3) followed by different letters in the same row are significantly different according to a mean analysis (Student t-test) and an analysis of variance (One-way ANOVA; Tukey's test, $p \leq 0.05$). MIC: minimum inhibitory concentration; Rs: resistance.

Several studies have been devoted to the control of pathogenic and phytopathogenic fungi in general, and *A. niger*, *A. flavus*, *F. oxysporum* and *C. albicans* in particular, through the use of various bioactive substances, either natural or synthetic. The results of this study are opposite of those of another study [49], in which sesquiterpenes of *J. thurifera* EOs did not present any antifungal activity against *C. albicans* CECT;1394. Similarly, in another study [28] the EOs of *J. thurifera* twigs collected from the Eastern sector of the Middle Atlas Mountains of Morocco exhibited antifungal activities against *A. niger*, *Penicillium expansum*, and *Penicillium digitatum*. The results of the study presented here are consistent with those focused on substances of bacterial and fungal origin [24], which reported an isolate from *Bacillus* sp. Gn-A11-18 exhibiting antifungal activity of 31.33 ± 0.58 mm against *C. albicans*; ATCC 10231 and a percentage of inhibition of 29.66 ± 0.57% against *A. niger.* Similarly, results of another study [50] showed significant antifungal activity mainly against *Alternaria alternata*, *F. oxysporum*, *F. solani*, *Rhizoctonia solani* and *Verticillium dahlia* with percentage inhibitions ranging from 24 to 92.1%.

4. Conclusions

The results of this study indicated that the EOs extracted from *J. thurifera* had excellent antioxidant and antimicrobial potencies against clinically important drug-resistant microbes. These results are intriguing since they suggest that EOs extracted from *J. thurifera* could potentially be used as an alternative to traditional antioxidant antimicrobial treatments. However, prior to any prospective application of the studied EOs as natural medicines to control microorganisms, evaluation of the potential side effects on non-target organisms along with pre-clinical and clinical works on non-human primates and humans will be required.

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Comparative Study on Chemical Constituents of Medicinal and Non-Medicinal Parts of Flos *Abelmoschus manihot*, Based on Metabolite Profiling Coupled with Multivariate Statistical Analysis

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Abstract: According to Chinese Pharmacopoeia (2020 edition), Abelmoschi Corolla (AC) is the dried corolla of Flos *Abelmoschus manihot* (FAM). Market research has found that AC is often mixed with the non-medicinal parts in FAM, including calyx, stamen, and pistil. However, previous studies have not clarified the relationship between the medicinal and non-medicinal parts of FAM. In this study, in order to investigate whether there is any distinction between the medicinal and non-medicinal parts of FAM, the characterization of the constituents in calyx, corolla, stamen, and pistil was analyzed by UFLC-Triple TOF-MS/MS. Multivariate statistical analysis was used to classify and screen differential constituents between medicinal and non-medicinal parts of FAM, and the relative contents of differential constituents were compared based on the peak intensities. Results showed that 51 constituents in medicinal and non-medicinal parts of FAM were identified, and the fragmentation pathways to different types of constituents were preliminarily deduced by the fragmentation behavior of the identified constituents. Furthermore, multivariate statistical analysis revealed that the medicinal and non-medicinal parts of FAM differed significantly; 20 differential constituents were screened out to reveal the characteristics of metabolic differences. Among them, the relative contents of 19 differential constituents in the medicinal part were significantly higher than those in non-medicinal parts. This study could be helpful in the quality evaluation of AC as well as provide basic information for the improvement of the market standard of AC.

Keywords: Flos *Abelmoschus manihot*; medicinal part; non-medicinal parts; UFLC-Triple TOF-MS/MS; metabolite profiling; multivariate statistical analysis

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1. Introduction

Abelmoschi Corolla (AC) is derived from the dried corolla of Flos *Abelmoschus manihot* (FAM), which has the functions of eliminating dampness and heat, subduing swelling, and detoxicating [1]. It is a traditional Chinese medicine (TCM) with a long medicinal history in China [2]. The research of pharmacology showed that AC has multiple pharmacological activities, such as anti-inflammatory, antioxidant [3,4], antitumor [5], anticonvulsant, antidepressant, and neuroprotective activities [6,7], as well as therapeutic actions on renal tubular injury and diabetic nephropathy [8–10].

As the main raw material of the Chinese patent medicine Huangkui capsule, AC has significant medicinal value and a huge market demand. Market research found that commercial medicinal material of AC was often mixed with non-medicinal parts of FAM,

including calyx, stamen, and pistil. The reason for this phenomenon is that the non-medicinal parts of FAM are not removed during the collection and processing of corolla. However, the chemical constituents of non-medicinal parts of FAM were not characterized in previous studies, so the comparative study between medicinal and non-medicinal parts of FAM was not clear-cut. The mixing of non-medicinal parts may alter the composition of AC, reduce its quality stability, and then affect its efficacy. Therefore, it is of practical value to study the chemical constituents of medicinal and non-medicinal parts of FAM and reveal the characteristics of metabolic differences.

Because it combines the separation powers of liquid chromatography with the very sensitive detection qualities of mass spectrometry, the LC-MS equipment has been frequently employed in TCM research in recent years [11]. Among them, ultra-fast liquid chromatography coupled with triple quadrupole-time of flight tandem mass spectrometry (UFLC-Triple TOF-MS/MS) is efficient and rapid in the determination of the molecular weight and characteristic fragment ions, by which the structure of multiple constituents in TCM can be identified quickly [12]. Hence, the characterization of the constituents in calyx, corolla, stamen, and pistil was analyzed by UFLC-Triple TOF-MS/MS. We integrated metabolic profiling and multivariate statistical analysis to separate the medicinal and non-medicinal parts of FAM and to define their chemical markers. The strategy for comparative analysis on chemical constituents of medicinal and non-medicinal parts of FAM was shown in Figure 1. The study could determine the differential constituents of medicinal and non-medicinal parts of FAM, so as to provide basic data for standardizing the harvest and market standards of AC. Our investigation will not only contribute to the quality evaluation of AC, but also has great significance in the quality stability improvement of AC.

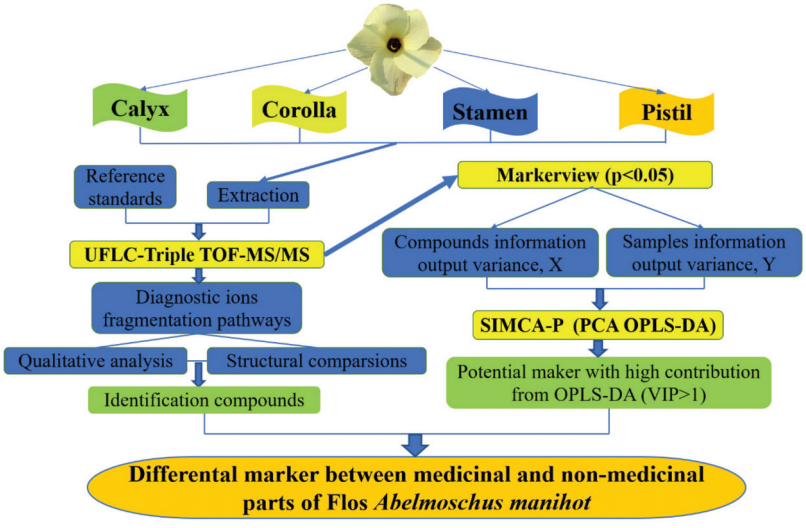

Figure 1. The strategy for comparative analysis on chemical constituents of medicinal and non-medicinal parts of Flos *Abelmoschus manihot* (FAM).

2. Materials and Methods

2.1. Chemicals and Reagents

The standard substances of 3,4,5-trihydroxybenzoic acid, rutin, hyperin, and quercetin were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 3,4-Dihydroxybenzoic acid was purchased from Shanghai Ronghe Pharmaceutical Technology Co., Ltd. (Shanghai, China). Chlorogenic acid, caffeic acid, and myricetin 3′-*O*-*β*-D-glucopyranoside were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Dihydromyricetin and myricetin

were purchased from Chengdu Aifa Bio-technology Co., Ltd. (Chengdu, China). Myricetin 3-*O*-*β*-D-glucopyranoside and quercetin 3-*O*-*β*-D-robinobioside were purchased from Liangwei Bio-technology Co., Ltd. (Nanjing, China). Quercetin 7-*O*-*β*-D-glucopyranoside, gossypetin 8-*O*-*β*-D-glucuronide, and quercetin 3-*O*-(6-*O*-acetyl-*β*-D-glucopyranoside) were purchased from Nanjing Casses Pharmaceutical Technology Co., Ltd. (Nanjing, China). Isoquercetin, quercetin 3′-*O*-*β*-D-glucoside, and tiliroside were purchased from Chengdu Chroma-Biotechnology Co., Ltd. (Chengdu, China). The purities of myricetin 3′-*O*-*β*-D-glucopyranoside was above 97% and other standards were greater than 98%, tested by HPLC analysis. Formic acid, acetonitrile, and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was applied to make deionized water.

2.2. Plant Materials

Five batches of FAM samples were collected from Xinghua City (Jiangsu Province, China 32°98′17″ N, 119°90′44″ E) in October 2019. Each batch was carefully divided into four parts, calyx, corolla, stamen, and pistil, which were separately dried in the oven. The drying temperature was set at 50 °C. The samples were authenticated by Professor Xunhong Liu as the flower of *Abelmoschus manihot* (L.) Medic. of Malvaceae family and the voucher specimens were deposited in the laboratory of Chinese medicine identification, Nanjing University of Chinese Medicine. The voucher numbers of the samples were as follows: 190923CA1−190923CA5 (calyx), 190923CO1−190923CO5 (corolla), 190923ST1−190923ST5 (stamen), 190923PI1−190923PI5 (pistil).

2.3. UFLC-Triple TOF-MS/MS Analysis

2.3.1. Preparation of Standard and Sample Solutions

A mixed standard stock solution of 18 standard substances was prepared with 70% (*v*/*v*) methanol. The diluted solutions were stored at 4 °C for further UFLC-Triple TOF-MS/MS analysis.

The 0.5 g of calyx, corolla, stamen, and pistil powder were properly weighed and ultrasonically extracted with 20 mL 70% (*v*/*v*) methanol for 30 min, respectively. To compensate for the weight lost during extraction, the same solvent was added after cooling to room temperature. The extract was then filtered, and the filtrate was centrifuged at 12,000 rpm/min for 10 min. Afterwards, the supernatant was filtered via a 0.22 μm membrane before UFLC-Triple TOF-MS/MS analysis.

2.3.2. UFLC-Triple TOF-MS/MS Conditions

The chromatographic analysis was performed on an UFLC-20AD XR system (Shimadzu, Kyoto, Japan). The separation was conducted by an Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) at 35 °C. The mobile phase contained 0.1% (*v*/*v*) aqueous formic acid water solution (A)–methanol:acetonitrile (1:1) (B) with the gradient elution: 0−3 min, 2% B; 3−10 min, 2−15% B; 10−14 min, 15−18% B; 14−20 min, 18−21% B; 20−30 min, 21−23% B; 30−45 min, 23−27% B; 45−50 min, 27−40% B; 50−52 min, 40−80% B; 52−54 min, 80−95% B. The injection volume was 10 μL and the flow rate was 1 mL/min.

A Triple TOF™ 5600 System MS/MS High Resolution Quadrupole Time-of-Flight Mass Spectrometer (AB SCIEX, Framingham, MA, USA) equipped with an electrospray ionization source was used for MS analysis in both positive and negative ion modes. The MS conditions were optimized as follows: the ion source temperature, 550 °C; the flow rate of curtain gas, 40 L/min; the flow rate of nebulization gas, 55 L/min; the flow rate of auxiliary gas, 55 L/min; the spray voltage, 4500 V in positive ion mode and -4500 V in negative ion mode; the declustering voltage, 100 V in positive ion mode and -100 V in negative ion mode. TOF MS and TOF MS/MS were scanned with the mass range of *m*/*z* 100−2000 and 50−1500, respectively.

2.3.3. Identification of the Constituents

A database of the chemical constituents of AC was formed based on previous research and the data were imported into the PeakView Software V.1.2 (AB SCIEX, Framingham, MA, USA). The chemical constituents of different parts of FAM were comprehensively characterized by comparing the retention time (t_R), accurately measuring mass and multi-stage MS/MS fragmentation information with standard substances, databases, and related literatures.

2.4. Multivariate Statistical Analysis

The data of UFLC-Triple TOF-MS/MS were processed by PeakView Software V.1.2 (AB SCIEX, Framingham, MA, USA) and MarkerView 1.2.1 software (AB Sciex). Principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS–DA) were performed using SIMCA-P 13.0 software (Umetrics AB, Umea, Sweden). PCA was used to categorize and identify different parts of FAM. OPLS-DA was performed to differentiate medicinal part and non-medicinal parts of FAM, as well as to identify the common differential constituents that cause the differences in each group of comparison by variable importance in the projection (VIP).

2.5. Relative Content Comparison of Differential Constituents

The relative contents of differential constituents in medicinal and non-medicinal parts of FAM were compared according to the peak intensities. To visualize and validate the distribution regularity of differential constituents among medicinal and non-medicinal parts of FAM, one-way analysis of variance (one-way ANOVA) was applied. Diagram of relative content comparison was charted by GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Identification of the Constituents in Medicinal and Non-Medicinal Parts of FAM

It was found that the best analytical selectivity and sensitivity was obtained in the negative ionization mode by comparing the data acquired in the two ion modes. As a result, we decided to collect data in the negative ion mode. The base peak chromatograms (BPCs) of the calyx (Figure 2A), corolla (Figure 2B), stamen (Figure 2C), and pistil (Figure 2D) extract in the negative ion mode are shown in Figure 2. Eventually, 51 constituents were identified, including 43 flavonoids, 6 organic acids, 1 ester, and 1 alkaloid. A total of 18 constituents were clearly identified by comparison with reference standards. Detailed information of the characteristic constituents is summarized in Table 1, with their structures presented in Figure S1.

3.1.1. Identification of Flavonoids

As the main active substance of AC, flavonoids have always been the research hotspot. In this study, a total of 43 flavonoids were identified from the extract of calyx, corolla, stamen, and pistil, including flavone, flavonols, and dihydroflavonol. At the same time, flavonols can be carefully divided into hibiscus parent flavonols, gossypetin parent flavonols, myricetin parent flavonols, quercetin parent flavonols, and kaempferol parent flavonols, respectively. The common substituents group on the A and B rings in flavonoids were hydroxyl, methoxy, and acetyl, and it was also extremely common for saccharides or glucuronic acids to interact with hemiacetal hydroxyl groups to form flavonoid glycosides. The basic fracture paths of flavonoids were the loss of these neutral pieces and Retro-Diels-Alder (RDA) cleavage of the C ring [13]. Figure 3 depicts several RDA cleavage mechanisms of related flavonoids. The molecular ion peak intensity of flavonoid glycosides was often modest, and the base peak was frequently the fragment peak of aglycon.

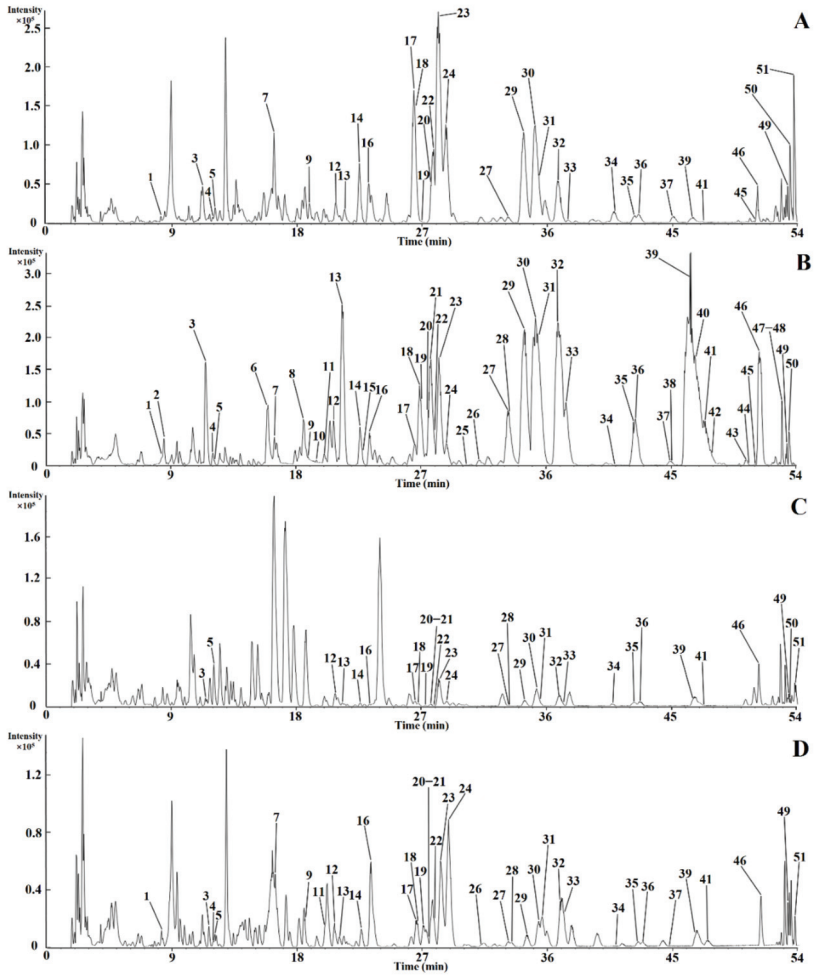

Figure 2. The base peak chromatograms (BPCs) of the calyx (**A**), corolla (**B**), stamen (**C**), and pistil (**D**) extract in negative ion mode. Note: the peak numbers denoted were the same as those in Table 1.

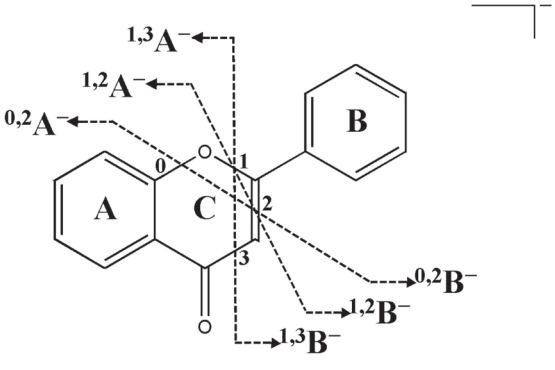

Figure 3. Schematic diagram of the fracture site of related flavonoids in negative ion mode.

Dihydroflavonol and flavone Compounds **11** and **34** were identified as dihydroflavonol and flavone, respectively. Compound **11** produced a $[M-H]^-$ ion at m/z 319.05 and abundance fragment ions, such as ions at m/z 301.06, 193.01, 165.02, and 151.00. The product ion at m/z 301.06 was generated from the elimination of H_2O from the molecular ion. The m/z 193.01 was the basic peak due to the removal of the B ring. The m/z 165.02 and 151.00 were $^{0,2}A^-$ (A fragment with A ring after the 0,2 bonds of C ring were broken) and $^{1,3}A^-$ (A fragment with A ring after the 1,3 bonds of C ring were broken). Therefore, compound **11** was identified as dihydromyricetin and further confirmed by the reference substance. The molecular ion peak of compound **34** was generated at m/z 593.15, suggesting that the molecular formula of the compound was $C_{27}H_{30}O_{15}$ and abundant fragment ions were generated at 285.04, 255.03, and 227.04. After removing the glycosyl and CH_2 from m/z 593.15 ion, the product ion of m/z 285.04 was generated. The fragment ions of m/z 255.03 and 227.04 were formed by dropping CH_2O and CO. Hence, compound **34** was proposed as 4′-methoxyl-5,7-dihydroxyl flavone-[-*O*-*β*-D-xylopyranosyl-(1→3)]-*O*-*β*-D-glucopyranoside, consistent with previous studies [14].

Hibiscus parent flavonols Compounds **6**, **8**, and **28** were identified as hibiscus parent flavonols with the adducted ion as $[M-H]^-$ or $[M+HCOO]^-$. The primary distinctive fragment ions of hibiscus parent flavonols were $^{1,2}A^-$ (A fragment with A ring after the 1,2 bonds of C ring were broken) and $^{1,3}A^-$ obtained by RDA fragmentation. Compounds **6** and **8** produced $[M+HCOO]^-$ ion at m/z 541.08 and abundance fragment ions, such as ions at m/z 333.03, 315.01, 287.02, 195.00, and 167.00. The m/z 333.03 was the product ion $[M-H-glc]^-$ due to cleavage of the glycosidic bond. The product ions at m/z 315.01 and 287.02 were generated from the elimination of H_2O and CO from m/z 333.03 ion. The m/z 195.00 and 167.00 were $^{1,2}A^-$ and $^{1,3}A^-$ obtained by RDA fragmentation. The adducted ion of compound **28** was observed as $[M-H]^-$ and the fragment ions basic peak $[M-H-glu]^-$ was observed at m/z 333.03 by the loss of 176 Da. Meanwhile, the m/z 195.00 and 167.00 were $^{1,2}A^-$ and $^{1,3}A^-$ obtained by RDA fragmentation. Therefore, compounds **6**, **8**, and **28** were identified as hibiscetin-3-*O*-glucoside, floramanoside B, and floramanoside C, respectively, which is consistent with previous studies [15]. See Table 1 for details.

Gossypetin and myricetin parent flavonols 7 and 11 constituents were identified as flavonoids with gossypetin and myricetin parent flavonols, respectively, including **12**, **13**, **25**, **39**, **40**, **43**, **44** and **14**, **15**, **16**, **18**, **19**, **20**, **21**, **26**, **33**, **38**, **41**. We found that gossypetin and myricetin parent flavonols were more likely to lose H_2O and CO fragments. In addition, the main characteristic fragments of these two flavonols were ions obtained from the cleavage of RDA. Taking compounds **39** and **20** as examples, the molecular ions were located at m/z 493.06 and 479.08, suggesting that the molecular formulas of the two compounds were $C_{21}H_{18}O_{14}$ and $C_{21}H_{20}O_{13}$, respectively. They all have base peaks at m/z 317.03, indicating that compounds **39** and **20** had a glycosidic acid and a glucose group, respectively. The main difference between the two was that the former fragment ions were $[^{1,2}A]^-$ (m/z 195.00), $[^{1,3}A]^-$ (m/z 167.00), and $[^{1,3}A-CO]^-$ (m/z 139.00), while the latter fragment ions were $[^{1,2}A]^-$ (m/z 179.00), $[^{1,3}A]^-$ (m/z 151.00), and $[^{1,2}B]^-$ (m/z 137.02). The difference is due to the different positions of hydroxyl groups in aglycon. Therefore, compounds **39** and **20** were identified as gossypetin 8-*O*-*β*-D-glucuronide and myricetin 3-*O*-*β*-D-glucopyranoside and further confirmed by the reference substance.

Quercetin parent flavonols 17 constituents including **17**, **22**, **23**, **24**, **27**, **29**, **30**, **31**, **32**, **35**, **37**, **42**, **45**, **46**, **47**, **48**, and **49** were identified as flavonoids with quercetin parent flavonols. They were more likely to lose H_2O and CO fragments and the ions obtained by RDA fragmentation were the main characteristic fragments. Taking compound **27** as example, the molecular ions was located at m/z 463.09, suggesting that the molecular formulas was $C_{21}H_{20}O_{12}$. The m/z 301.03 was the product ion $[M-H-glc]^-$ due to cleavage of the glycosidic bond. The fragments ions of m/z 253.05 and 237.54 were formed by dropping CO and H_2O. The m/z 179.00 and 151.00 were $^{1,2}A^-$ and $^{1,3}A^-$ obtained by RDA fragmentation. Therefore, compound **27** was identified as isoquercitrin and confirmed by the reference substance. The specific fragment information was shown in Table 1.

Kaempferol parent flavonols Compounds **36**, **50**, and **51** were identified as kaempferol parent flavonols. Taking compound **50** as an example, the molecular ion was located at m/z 593.13, suggesting that the molecular formula was $C_{30}H_{26}O_{13}$. The m/z 447.09 was the fragments ion $[M-H-C_9H_6O_2]^-$ by dropping hydroxycinnamoyl group, m/z 285.04 was the product ion $[M-H-C_9H_6O_2-glc]^-$ due to the cleavage of the glycosidic bond. The fragments ions of m/z 257.05 and 239.03 were ions $[M-H-C_9H_6O_2-glc-CO]^-$ and $[M-H-C_9H_6O_2-glc-CO-H_2O]^-$ formed by dropping CO and H_2O. Therefore, compound **50** was identified as tiliroside which confirmed by the reference substance.

3.1.2. Identification of Organic Acids

The adducted ion of organic acids was observed as $[M-H]^-$ in the negative mode. The MS/MS spectra of compounds **1**, **4**, **9** usually had a basic peak at $[M-H-CO_2]^-$, and then produce $[M-H-CO_2-H_2O]^-$ by the loss of H_2O. The basic peak of compound **7** was the removal of caffeic acid group $[M-H-C_9H_6O_3]^-$, followed by the loss of $2H_2O$ and CO produce $[M-H-C_9H_6O_3-2H_2O-CO]^-$. Compounds **1**, **4**, **7**, **9** were identified as 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, chlorogenic acid, and caffeic acid, respectively, which were further confirmed by the reference substance. The basic peak of compounds **2**, **3** were $[M-H-glc]^-$. At the same time, the other fragments were consistent with the identified components **1**, **4**. Therefore, compounds **2**, **3** were identified as gallic acid 3-*O*-*β*-glucoside and protocatecheuic acid 3-*O*-*β*-D-glucoside. See Table 1 for details.

3.1.3. Identification of Ester

Compound **5** gave precursor ion $[M-H]^-$ at m/z 299.08, suggesting that its molecular formula was $C_{13}H_{16}O_8$. The fragment ion was observed at m/z 137.02 by the loss of 162 Da, attributed to the loss of a glucose group. The loss of H_2O from $[M-H-glc]^-$ resulted in the fragment at m/z 119.03, showing the existence of hydroxyl group. Hence, it was identified as 4-hydroxybenzoic acid *β*-D-glucosyl ester by referring to the previous studies [16].

3.1.4. Identification of Alkaloid

Compound **10** gave precursor ion $[M-H]^-$ at m/z 252.09, suggesting that its molecular formula was $C_{12}H_{15}NO_5$. The fragment ions basic peak $[M-H-H_2O]^-$ was observed at m/z 234.08 by the loss of 18 Da, indicating the presence of hydroxyl group. Afterwards, the loss of CO_2 from $[M-H-H_2O]^-$ produced the fragment at m/z 190.09. Thus, it was identified as acortatarine A, consistent with previous studies [17].

3.2. Multivariate Statistical Analysis

PCA was conducted to classify the different parts of FAM. The first two principal components accounted for more than 75% of the total variance, could be used to represent overall information of samples (R^2X [1] = 0.554, R^2X [2] = 0.235). The PCA scores plot indicated that the medicinal part and non-medicinal parts of FAM were divided into two clusters (Figure 4). Corolla were gathered in the positive axis, non-medicinal parts of FAM were distributed in the negative axis, indicating that there was a significant difference between the medicinal and non-medicinal parts of FAM.

OPLS-DA was used to further distinguish the medicinal and non-medicinal parts of FAM, and to find out the important constituents that cause the differences with VIP values. The OPLS-DA score scatter plot, VIP plot, and S-Plot for comparison of the medicinal and non-medicinal parts of FAM were shown in Figure 5A–C. The OPLS-DA model demonstrated good adaptability (R^2X = 0.938, 0.949, and 0.937, respectively, R^2Y = 0.999, 0.999, and 0.999, respectively) and predictability (Q^2 = 0.999, 0.998, and 0.998, respectively). Calyx and corolla, stamen and corolla, pistil and corolla were all separated into two clusters along PC1 axis. The result revealed that the difference between the medicinal and non-medicinal parts of FAM was significant, which was completely consistent with the result of PCA.

Table 1. Identification of 51 constituents in calyx, corolla, stamen, and pistil by UFLC-Triple TOF-MS/MS.

No.	t_R (min)	Molecular Formula	MS^1 (*m*/*z*)	MS^2 (*m*/*z*)	Error (ppm)	Compound	Calyx	Corolla	Stamen	Pistil	References
1 *	8.29	$C_7H_6O_5$	169.0150[M−H]$^-$	125.02[M−H−CO_2]$^-$, 107.01[M−H−CO_2−H_2O]$^-$	4.73	3,4,5-Trihydroxybenzoic acid	+	+	−	+	[18–22]
2	8.49	$C_{13}H_{16}O_{10}$	331.0680[M−H]$^-$	169.01[M−H−glc]$^-$, 125.02[M−H−glc−CO_2]$^-$, 107.01[M−H−glc−CO_2−H_2O]$^-$	2.81	Gallic acid 3-*O*-*β*-glucoside	−	+	−	−	[21]
3	11.43	$C_{13}H_{16}O_9$	315.0730[M−H]$^-$	153.02[M−H−glc]$^-$, 109.03[M−H−glc−CO_2]$^-$, 91.02[M−H−glc−CO_2−H_2O]$^-$	2.67	Protocatecheuic acid 3-*O*-*β*-D-glucoside	+	+	+	+	[23]
4 *	11.99	$C_7H_6O_4$	153.0194[M−H]$^-$	109.03[M−H−CO_2]$^-$, 91.02[M−H−CO_2−H_2O]$^-$	0.46	3,4-Dihydroxybenzoic acid	+	+	−	+	[21–23]
5	12.03	$C_{13}H_{16}O_8$	299.0782[M−H]$^-$	137.02[M−H−glc]$^-$, 119.03[M−H−glc−H_2O]$^-$	3.21	4-Hydroxybenzoic acid *β*-D-glucosyl ester	+	+	+	+	[16]
6	15.99	$C_{21}H_{20}O_{14}$	541.0838[M+HCOO]$^-$	495.08[M−H]$^-$, 333.03[M−H−glc]$^-$, 315.01[M−H−glc−H_2O]$^-$, 287.02[M−H−glc−H_2O−CO]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$	2.57	Hibiscetin-3-*O*-glucoside	−	+	−	−	[24,25]
7 *	16.41	$C_{16}H_{18}O_9$	353.0881[M−H]$^-$	191.05[M−H−$C_9H_6O_3$]$^-$, 127.04[M−H−$C_9H_6O_3$−2H_2O−CO]$^-$	0.82	Chlorogenic acid	+	+	−	+	[21,26]
8	18.56	$C_{21}H_{20}O_{14}$	541.0835[M+HCOO]$^-$	495.08[M−H]$^-$, 333.03[M−H−glc]$^-$, 315.01[M−H−glc−H_2O]$^-$, 287.02[M−H−glc−H_2O−CO]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$	2.01	Floramanoside B	−	+	−	−	[15,27]
9 *	18.75	$C_9H_8O_4$	179.0353[M−H]$^-$	135.04[M−H−CO_2]$^-$	1.79	Caffeic acid	+	+	−	+	[21]
10	19.41	$C_{12}H_{15}NO_5$	252.0887[M−H]$^-$	234.08[M−H−H_2O]$^-$, 190.09[M−H−H_2O−CO_2]$^-$	3.95	Acortatarine A	−	+	−	−	[17]
11 *	20.29	$C_{15}H_{12}O_8$	319.0466[M−H]$^-$	301.06[M−H−H_2O]$^-$, 193.01[M−H−B ring]$^-$, 165.02[^{0,2}A]$^-$, 151.00[^{1,3}A]$^-$	2.07	Dihydromyricetin	−	+	−	+	[28,29]
12	20.72	$C_{27}H_{28}O_{19}$	655.1151[M−H]$^-$	479.08[M−H−glu]$^-$, 317.03[M−H−glu−glc]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$, 139.00[^{1,3}A−CO]$^-$	−0.15	Gossypetin 3-*O*-*β*-glucopyranoside-8-*O*-*β*-glucuronopyranoside	+	+	+	+	[24]
13	21.23	$C_{27}H_{28}O_{19}$	655.1160[M−H]$^-$	479.08[M−H−glu]$^-$, 317.03[M−H−glu−glc]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$, 139.00[^{1,3}A−CO]$^-$	1.22	Gossypetin 3-*O*-*β*-glucuronopyranoside-8-*O*-*β*-glucopyranoside	+	+	+	+	[24]
14	22.5	$C_{26}H_{28}O_{17}$	611.1251[M−H]$^-$	317.03[M−H−gal−xyl]$^-$, 271.02[M−H−gal−xyl−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	−0.44	Floramanoside A	+	+	+	+	[15]
15	22.89	$C_{21}H_{20}O_{13}$	479.0827[M−H]$^-$	317.03[M−H−gal]$^-$, 271.02[M−H−gal−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	−0.86	Myricetin 7-*O*-*β*-D-galactopyranoside	−	+	−	−	[30,31]

Table 1. *Cont.*

No.	t_R (min)	Molecular Formula	MS^1 (*m*/*z*)	MS^2 (*m*/*z*)	Error (ppm)	Compound	Calyx	Corolla	Stamen	Pistil	References
16	23.17	$C_{26}H_{28}O_{17}$	611.1272[M−H]$^-$	317.03[M−H−glc−xyl]$^-$, 271.02[M−H−glc−xyl−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	2.99	Myricetin 3-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucopyranoside	+	+	+	+	[24]
17	26.5	$C_{32}H_{38}O_{20}$	741.1904[M−H]$^-$	301.03[M−H−gal−rha−xyl]$^-$, 271.02[M−H−gal−rha−xyl−CO]$^-$, 255.03[M−H−gal−rha−xyl−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	2.74	quercetin 3-O[β-D-xylopyranosyl (1→2)-α-L-rhamnopyranosyl (1→6) -β-D-galactopyranoside	+	+	+	+	[30]
18	26.86	$C_{21}H_{20}O_{13}$	479.0832[M−H]$^-$	317.03[M−H−gal]$^-$, 271.02[M−H−gal−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	0.19	Myricetin 3-*O*-*β*-D-galactopyranoside	+	+	+	+	[30]
19	27.05	$C_{27}H_{30}O_{17}$	625.1415[M−H]$^-$	317.03[M−H−gal−rha]$^-$, 271.02[M−H−gal−rha−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	0.77	Myricetin 3-robinobioside	+	+	+	+	[30,31]
20 *	27.52	$C_{21}H_{20}O_{13}$	479.0838[M−H]$^-$	317.03[M−H−glc]$^-$, 271.02[M−H−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	1.44	Myricetin 3-*O*-*β*-D-glucopyranoside	+	+	+	+	[24,30]
21	27.53	$C_{27}H_{30}O_{17}$	625.1425[M−H]$^-$	317.03[M−H−glc−rha]$^-$, 271.02[M−H−glc−rha−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	2.37	Myricetin 3-*O*-rutinose	–	+	+	+	[31]
22	27.95	$C_{28}H_{32}O_{16}$	623.1600[M−H]$^-$	315.05[M−H−gal−rha]$^-$, 271.02[M−H−gal−rha−CO_2]$^-$	−2.82	Floramanoside D	+	+	+	+	[15,27]
23	28.23	$C_{26}H_{28}O_{16}$	595.1308[M−H]$^-$	301.03[M−H−gal−xyl]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	0.57	Quercetin 3-*O*-*β*-D-xylopyranosyl-(1→2)-*O*-*β*-D-galactopyranoside	+	+	+	+	[32]
24	28.79	$C_{26}H_{28}O_{16}$	595.1294[M−H]$^-$	301.03[M−H−glc−xyl]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−1.78	Quercetin 3-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucopyranoside	+	+	+	+	[32]
25	30.28	$C_{21}H_{20}O_{13}$	479.0830[M−H]$^-$	317.03[M−H−glc]$^-$, 271.02[M−H−glc−CO−H_2O]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$, 139.00[^{1,3}A−CO]$^-$	−0.23	Gossypetin 3-*O*-*β*-D-glucopyranoside	–	+	–	–	[25]
26	31.17	$C_{23}H_{22}O_{14}$	521.0930[M−H]$^-$	479.08[M−H−C_2H_2O]$^-$, 317.03[M−H−C_2H_2O−gal]$^-$, 271.02[M−H−C_2H_2O−gal−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	−1.30	Myricetin 3-*O*-(6-*O*-acetyl-*β*-D-galactopyranoside)	–	+	–	+	[30,31]
27 *	33.07	$C_{21}H_{20}O_{12}$	463.0883[M−H]$^-$	301.03[M−H−glc]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	0.22	Quercetin 7-*O*-*β*-D-glucopyranoside	+	+	+	+	[33]
28	33.18	$C_{21}H_{18}O_{15}$	509.0573[M−H]$^-$	333.03[M−H−glu]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	0.02	Floramanoside C	–	+	+	+	[15,27]

Table 1. *Cont.*

No.	t_R (min)	Molecular Formula	MS^1 (*m*/*z*)	MS^2 (*m*/*z*)	Error (ppm)	Compound	Calyx	Corolla	Stamen	Pistil	References
29 *	34.19	$C_{27}H_{30}O_{16}$	609.1455[M−H]$^-$	301.03[M−H−rha−gal]$^-$, 271.02[M−H−rha−gal−CO]$^-$, 255.03[M−H−rha−gal−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−1.00	Quercetin 3-*O*-*β*-D-robinobioside	+	+	+	+	[30]
30 *	35.1	$C_{21}H_{20}O_{12}$	463.0883[M−H]$^-$	301.03[M−H−gal]$^-$, 271.02[M−H−gal−CO]$^-$, 255.03[M−H−gal−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	0.22	Hyperin	+	+	+	+	[26,34]
31 *	35.55	$C_{27}H_{30}O_{16}$	609.1461[M−H]$^-$	301.03[M−H−rha−glc]$^-$, 271.02[M−H−rha−glc−CO]$^-$, 255.03[M−H−rha−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−0.02	Rutin	+	+	+	+	[24,26]
32 *	36.63	$C_{21}H_{20}O_{12}$	463.0877[M−H]$^-$	301.03[M−H−glc]$^-$, 271.02[M−H−glc−CO]$^-$, 255.03[M−H−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−1.08	Isoquercitrin	+	+	+	+	[30,34]
33 *	37.38	$C_{21}H_{20}O_{13}$	479.0815[M−H]$^-$	317.03[M−H−glc]$^-$, 271.02[M−H−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	−3.36	Myricetin 3′-*O*-*β*-D-glucopyranoside	+	+	+	+	[25,30]
34	40.94	$C_{27}H_{30}O_{15}$	593.1506[M−H]$^-$	285.04[M−H−glc−xyl−CH_2]$^-$, 255.03[M−H−glc−xyl−CH_2−CH_2O]$^-$, 227.04[M−H−glc−xyl−CH_2− CH_2O −CO]$^-$	−0.99	4′-Methoxyl-5,7-dihydroxyl flavone-[-*O*-*β*-D-xylopyranosyl-(1→3)]-*O*-*β*-D-glucopyranoside	+	+	+	+	[16]
35	42.23	$C_{23}H_{22}O_{13}$	505.0981[M−H]$^-$	463.09[M−H−C_2H_2O]$^-$, 301.03[M−H−C_2H_2O−gal]$^-$, 271.02[M−H−C_2H_2O−gal−CO]$^-$, 255.03[M−H−C_2H_2O−gal−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−1.31	6″-Acetylhyperin	+	+	+	+	[30]
36	42.8	$C_{21}H_{20}O_{11}$	447.0932[M−H]$^-$	285.04[M−H−glc]$^-$, 255.03[M−H−glc−CHO]$^-$, 227.03[M−H−glc−CHO−CO]$^-$ 445.08[M−H−C_2H_2O−H_2O]$^-$,	−0.20	kaempferol 3-*O*-*β*-D-glucoside	+	+	+	+	[35]
37	44.89	$C_{23}H_{22}O_{13}$	505.0985[M−H]$^-$	301.03[M−H−C_2H_2O−glc]$^-$, 271.02[M−H−C_2H_2O−glc−CO]$^-$, 255.03[M−H−C_2H_2O−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−0.51	7-*O* Acetyl Isoquercitrin	+	+	−	+	[30]

Table 1. *Cont.*

No.	t_R (min)	Molecular Formula	MS^1 (*m*/*z*)	MS^2 (*m*/*z*)	Error (ppm)	Compound	Calyx	Corolla	Stamen	Pistil	References
38	44.9	$C_{23}H_{22}O_{14}$	521.0932[M−H]$^-$	479.08[M−H−C_2H_2O]$^-$, 317.03[M−H−C_2H_2O−glc]$^-$, 271.02[M−H−C_2H_2O−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	−0.92	Myricetin 3′-*O*-(6-*O*-acetyl-*β*-D-glucopyranoside)	−	+	−	−	[30]
39 *	46.64	$C_{21}H_{18}O_{14}$	493.0621[M−H]$^-$	317.03[M−H−glu]$^-$, 271.02[M−H−glu−CO−H_2O]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$, 139.00[^{1,3}A−CO]$^-$	−0.57	Gossypetin 8-*O*-*β*-D-glucuronide	+	+	+	+	[24,25]
40	46.98	$C_{15}H_{10}O_8$	317.0306[M−H]$^-$	271.02[M−H−CO−H_2O]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$, 139.00[^{1,3}A−CO]$^-$	0.98	Gossypetin	−	+	−	−	[24]
41 *	47.4	$C_{15}H_{10}O_8$	317.0309[M−H]$^-$	271.02[M−H−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 137.02[^{1,2}B]$^-$	1.92	Myricetin	+	+	+	+	[24,31]
42 *	47.93	$C_{23}H_{22}O_{13}$	505.0975[M−H]$^-$	463.09[M−H−C_2H_2O]$^-$, 301.03[M−H−C_2H_2O−glc]$^-$, 271.02[M−H−C_2H_2O−glc−CO]$^-$, 255.03[M−H−C_2H_2O−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−2.49	Quercetin 3-*O*-(6-*O*-acetyl-*β*-D-glucopyranoside)	−	+	−	−	[30]
43	50.34	$C_{21}H_{20}O_{13}$	479.0824[M−H]$^-$	317.03[M−H−glc]$^-$, 271.02[M−H−glc−CO−H_2O]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$, 139.00[^{1,3}A−CO]$^-$	−1.48	Gossypetin 3′-*O*-glucoside	−	+	−	−	[24]
44	50.67	$C_{23}H_{22}O_{14}$	521.0930[M−H]$^-$	317.03[M−H−glu−C_2H_4]$^-$, 299.02[M−H−glu−C_2H_4−H_2O]$^-$, 195.00[^{1,2}A]$^-$, 167.00[^{1,3}A]$^-$	−1.30	Floramaroside F	−	+	−	−	[15,24]
45	51.04	$C_{21}H_{20}O_{11}$	447.0923[M−H]$^-$	301.03[M−H−rha]$^-$, 271.02[M−H−rha−CO]$^-$, 255.03[M−H−rha−CO−H_2O]$^-$, 151.00[^{1,3}A]$^-$, 179.00[^{1,2}A]$^-$	−2.21	Quercetin 3-*O*-*α*-L-rhamnopyranoside	+	+	−	−	[36,37]
46 *	51.28	$C_{21}H_{20}O_{12}$	463.0877[M−H]$^-$	301.03[M−H−glc]$^-$, 273.04[M−H−glc−CO]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−1.08	Quercetin 3′-*O*-*β*-D-glucoside	+	+	+	+	[24]
47	52.91	$C_{21}H_{18}O_{13}$	477.0674[M−H]$^-$	301.03[M−H−glu]$^-$, 151.00[^{1,3}A]$^-$, 179.00[^{1,2}A]$^-$	−0.13	Quercetin 3′-*O*-*β*-glucuronide	−	+	−	−	[26,38]
48	52.92	$C_{23}H_{22}O_{13}$	505.0983[M−H]$^-$	463.09[M−H−C_2H_2O]$^-$, 301.03[M−H−C_2H_2O−glc]$^-$, 271.02[M−H−C_2H_2O−glc−CO]$^-$, 255.03[M−H−C_2H_2O−glc−CO−H_2O]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$	−0.91	Floramaroside E	−	+	−	−	[15,39]

Table 1. *Cont.*

No.	t_R (min)	Molecular Formula	MS^1 (*m*/*z*)	MS^2 (*m*/*z*)	Error (ppm)	Compound	Calyx	Corolla	Stamen	Pistil	References
49 *	53.46	$C_{15}H_{10}O_7$	301.0363[M−H]$^-$	273.04[M−H−CO]$^-$, 179.00[^{1,2}A]$^-$, 151.00[^{1,3}A]$^-$, 107.01[^{1,2}A−CO−CO_2]$^-$	3.06	Quercetin	+	+	+	+	[13,24]
50 *	53.61	$C_{30}H_{26}O_{13}$	593.1279[M−H]$^-$	447.09[M−H−$C_9H_6O_2$]$^-$, 285.04[M−H−$C_9H_6O_2$−glc]$^-$, 257.05[M−H−$C_9H_6O_2$−glc−CO]$^-$, 239.03[M−H−$C_9H_6O_2$−glc−CO−H_2O]$^-$	−3.64	Tiliroside	+	+	+	−	[21]
51	53.98	$C_{32}H_{28}O_{14}$	635.1380[M−H]$^-$	285.04[M−H−$C_{16}H_{19}O_8$]$^-$	−4.14	3-*O*-kaempferol-3-*O*-acetyl-6-*O*-(*p*-coumaroyl)-*β*-D-glucopyranoside	+	−	+	+	[24]

Note: "*", comparison with standard substances; "+", detected; "−", not detected; "glc", glucose; "glu", glucuronic acid; "gal", galactose; "xyl", xylose; "rha", rhamnos. MS^1: quasi-molecular ion, MS^2: product fragment ion.

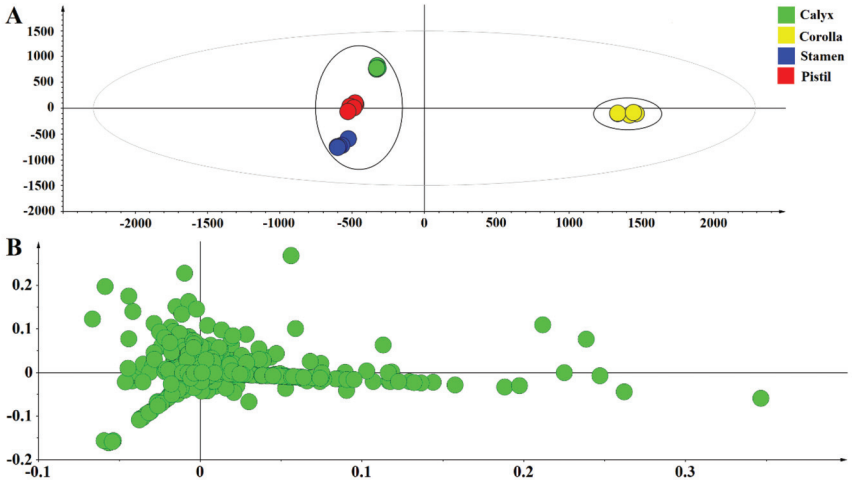

Figure 4. The principal component analysis scores scatter plot (**A**) and loading scatter plot (**B**) of medicinal and non-medicinal parts of FAM.

The identification of potential chemical markers to distinguish the medicinal and non-medicinal parts of FAM was the focus of this study. Based on their VIP values (i.e., larger than 1.0), 20 constituents were screened out to discriminate the medicinal and non-medicinal parts of FAM, including protocatecheuic acid 3-*O*-*β*-D-glucoside (**3**), hibiscetin-3-*O*-glucoside (**6**), floramanoside B (**8**), gossypetin 3-*O*-*β*-glucopyranoside-8-*O*-*β*-glucuronopyranoside (**12**), gossypetin 3-*O*-*β*-glucuronopyranoside-8-*O*-*β*-glucopyranoside (**13**), myricetin 3-*O*-*β*-D-galact opyranoside (**18**), myricetin 3-*O*-*β*-D-glucopyranoside (**20**), myricetin 3-*O*-rutinose (**21**), quercetin 3-*O*-*β*-D-xylopyranosyl-(1→2)-*O*-*β*-D-galactopyranoside (**23**), floramanoside C (**28**), quercetin 3-*O*-*β*-D-robinobioside (**29**), hyperin (**30**), rutin (**31**), isoquercitrin (**32**), myricetin 3′-*O*-*β*-D glucopyranoside (**33**), 6″-acetylhyperin (**35**), gossypetin 8-*O*-*β*-D-glucuronide (**39**), myricetin (**41**), quercetin 3′-*O*-*β*-D-glucoside (**46**), and floramaroside E (**48**). Therefore, these constituents could be selected as differential constituents to distinguish the medicinal and non-medicinal parts of FAM.

3.3. Relative Content Comparison of Differential Constituents

The relative contents of differential constituents in medicinal and non-medicinal parts of FAM were compared based on peak intensity. Furthermore, the one-way ANOVA followed by least significant difference test (variance homogeneity) or Tamhane's test (variance heterogeneity) was carried out to illustrate the abundance variation of 20 differential constituents. As shown in Figure 6. The relative contents of 19 differential constituents (protocatecheuic acid 3-*O*-*β*-D-glucoside, hibiscetin-3-*O*-glucoside, floramanoside B, gossypetin 3-*O*-*β*-glucopyranoside-8-*O*-*β*-glucuronopyranoside, gossypetin 3-*O*-*β*-glucuronopyranoside-8-*O*-*β*-glucopyranoside, myricetin 3-*O*-*β*-D-galactopyranoside, myricetin 3-*O*-*β*-D-glucopyranoside, myricetin 3-*O*-rutinose, floramanoside C, quercetin 3-*O*-*β*-D-robinobioside, hyperin, rutin, isoquercitrin, myricetin 3′-*O*-*β*-D glucopyranoside, 6″-acetylhyperin, gossypetin 8-*O*-*β*-D-glucuronide, myricetin, quercetin 3′-*O*-*β*-D-glucoside, floramaroside E) in corolla were significantly higher than those in non-medicinal parts, only quercetin 3-*O*-*β*-D-xylopyranosyl-(1→2)-*O*-*β*-D-galactopyranoside in calyx of non-medicinal part was higher than that of medicinal part.

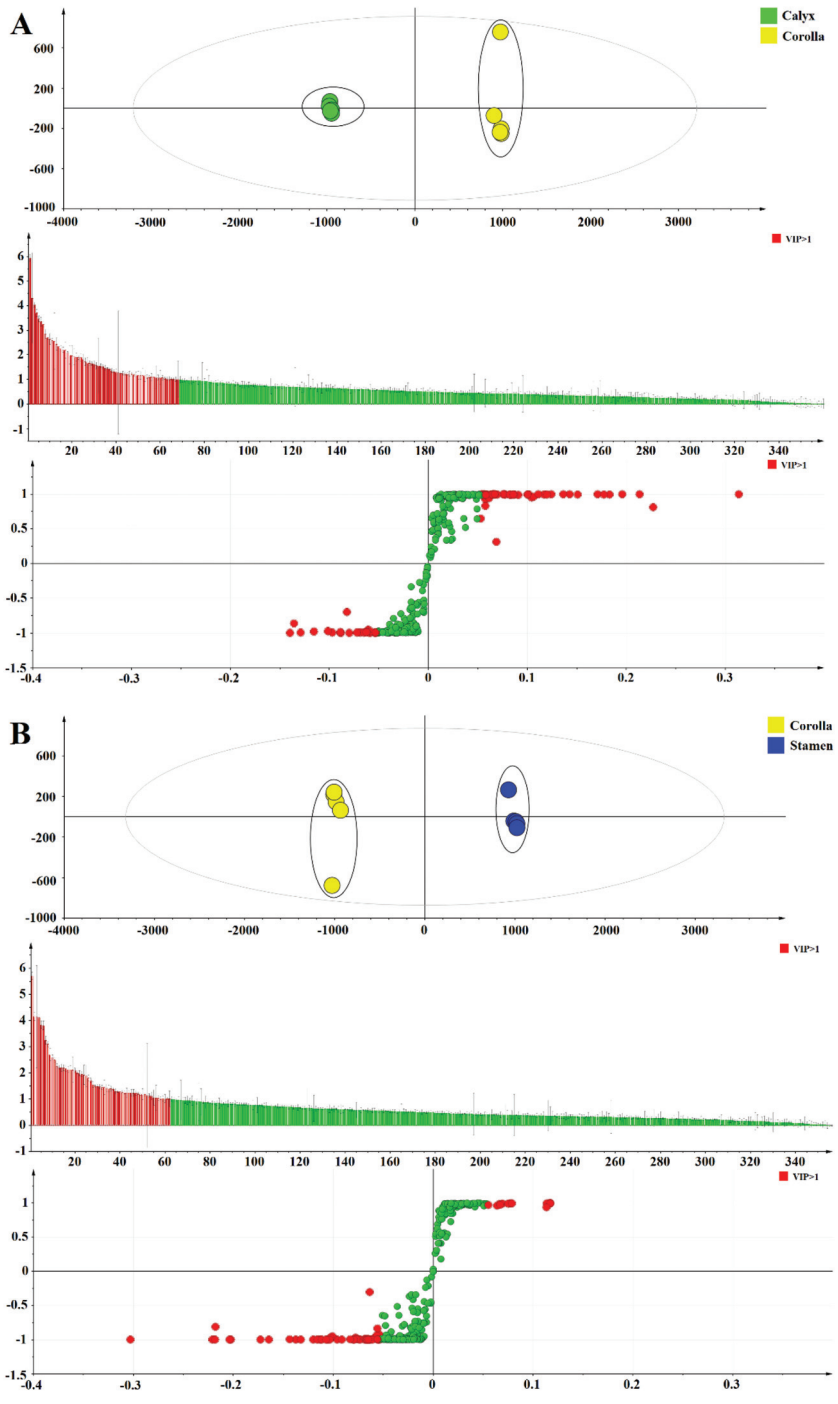

Figure 5. *Cont.*

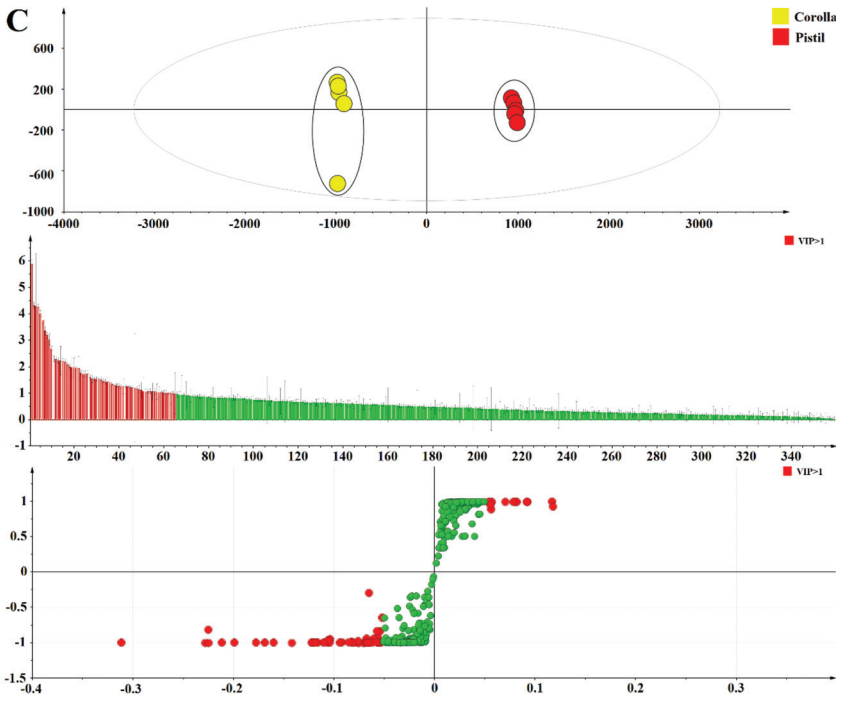

Figure 5. The orthogonal partial least squares discriminant analysis score scatter plot, VIP plot, and S-Plot of calyx and corolla (**A**), stamen and corolla (**B**), pistil and corolla (**C**).

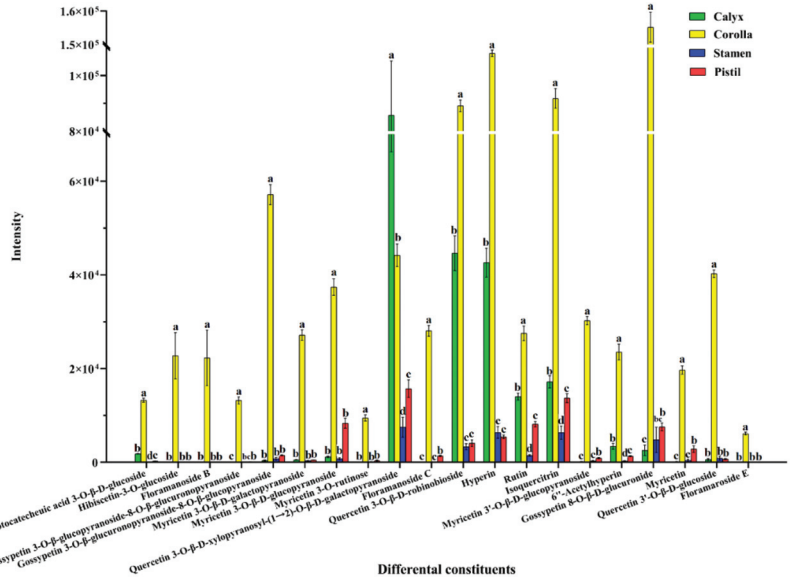

Figure 6. The relative contents of 20 differential constituents in medicinal and non-medicinal parts of FAM. (Different letters indicate significant differences, $p < 0.05$).

4. Discussion

The clinical efficacy of TCM is determined by its chemical constitution [40]. The Chinese Pharmacopoeia officially recorded that the medicinal part of AC was only the corolla of FAM. However, the phenomenon of doping non-medicinal parts in AC was common. Therefore, the chemical constituents of medicinal and non-medicinal parts of FAM were analyzed. A total of 20 differential constituents, including 1 organic acid and 19 flavonoids, were screened out by multivariate statistical analysis and their relative contents were compared. Among them, the relative content of 19 differential constituents in the corolla of FAM was significantly higher than that in the non-medicinal parts. Many studies have shown that the flavonoids of AC such as hyperin and isoquercitrin possess anti-inflammatory and renal injury protective properties [41–43], which is consistent with the clinical efficacy of AC [44,45]. Due to the great difference in chemical constituents between the medicinal part and the non-medicinal parts, the latter may not have the same therapeutic effect as the medicinal part. The screened differential constituents might be the pharmacodynamic substances of AC, which provide ideas for the research of pharmacodynamic substance basis of AC.

In summary, 51 constituents from medicinal and non-medicinal parts of FAM were identified and their metabolic profiles were compared. 20 differential constituents were screened to distinguish the medicinal part and non-medicinal parts of FAM. The great difference in the relative content of them indicates that the non-medicinal parts of FAM are hardly a substitute for the corolla part. Our study could be conducive to the quality evaluation and quality stability improvement of AC and provide a scientific basis for strictly regulating the harvest and market standards of AC.

Supplementary Materials: The following is available online at https://www.mdpi.com/article/10.3390/horticulturae8040317/s1, Figure S1. Chemical structures of constituents identified in medicinal and non-medicinal parts of Flos *Abelmoschus manihot*.

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Antioxidant Activity and Chemical Characteristics of *Sambucus nigra* L. Blossom from Different Regions in Bulgaria

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Abstract: The aim of the current study was to investigate some bioactive compounds from *Sambucus nigra* L. blossoms and to evaluate the antioxidant potential of the obtained extracts. In this study, samples from four different regions of Bulgaria /Rhodopes, Plovdiv, Strandzha and Dobrich region/ from *Sambucus nigra* L. were collected and analyzed for total phenols, flavonoids, sugars and amino acids. The antioxidant activity of the extracts was evaluated by four assays based on different mechanisms. The sweetness index and total sweetness index of the extracts were also evaluated. The carbohydrate composition of the leaves and the blossoms was determined, with glucose and fructose predominating in both cases, as their contents were not above 3%. Nineteen amino acids have been identified in the composition of *Sambucus nigra* L., and glutamic, leucine and asparagine acids are predominant. The highest antioxidant activity and total content of phenols (49.2 ± 1 mg GAE/g) and flavonols (18.6 ± 0.5 mgQE/g) were found in the sample from the Rhodope region. Therefore, the higher altitude and lower temperature in mountains could influence the accumulation of secondary metabolites in blossoms of *Sambucus nigra* L., which improves the antioxidant potential of the samples.

Keywords: *Sambucus nigra* L.; medicinal plants; antioxidant activity; flavonoids; phenols; carbohydrates; amino acids

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1. Introduction

Elder or black elder *(Sambucus nigra* L.) is a perennial, deciduous shrub or small tree with shallow roots, propagated by seed. The tree reaches up to 8–10 m in height and 20–30 cm in diameter. It blooms from May to June, depending on the altitude, with white-cream colored or greenish-yellow individual flowers gathered in flat, complex, umbrella-like umbels, approximately 10–20 cm in diameter, containing 3–5 small seeds. The blossoms are characterized by a fresh, intense, fruity-sweet aroma.

Black elderflower tea may help in a variety of cases, such as hoarse voice (dysphonia) and upper respiratory tract inflammation [1], hemorrhoids, ascites, high blood pressure, impotence, urinary tract inflammation, kidney and bladder inflammation, prostatitis, hematuria, shortness of breath (dyspnea), and obesity [2–4]. It has been found that elderflowers promote secretion from sweat glands, while elderberries stimulate renal activity. Up to this day, the blossoms of *Sambucus nigra* L. are used to treat diseases, such as fever, cold

and influenza infections [5]. In the composition of blossoms, a large number of bioactive ingredients can be found, including terpenes, sterols, polyphenols, proteins, vitamins and minerals [6–9].

The health benefits of different parts of the plant *Sambucus nigra* L. have been investigated by different authors [10,11] and the data are summarized in Table 1.

Table 1. Healing properties of *Sambucus nigra* L.

Parts of the Tree	Components' Properties
Blossoms	Promotes sweating, antipyretic agent, expectorant, anti-inflammatory agent, immunostimulant, antiviral and antibacterial activity;
Fruits	Strengthening the immune system, antineuralgic, antiviral, antibacterial action, antioxidant, laxative, diuretic agent;
Leaves	Diuretic agent, blood purifying properties, laxative agent, detoxifying properties;
Bark	Diuretic agent, laxative, inflammatory agent;

Polyphenolic compounds are chemical compounds with a positive effect on human health and various pharmacological effects: antiviral, antibacterial and anti-cancer functions [9]. Some publications have shown that elderflower extract contains bioactive compounds that are able to metabolize glucose and lipids, which leads to a reduction in fat accumulation [12,13]. Other studies have reported strong antimicrobial effects of elderflower on various nosocomial pathogens, especially on methicillin-resistant *Staphylococcus aureus* MRSA /clinically significant pathogen/ [14].

The healthy effects of *Sambucus nigra* L. on the human body based on the high antioxidants, bioactive flavonoids and phenolic acid content, mineral salts, and fibers, as well the aim to enrich a healthy diet with phytocomponents, motivates the research for the possibilities for inclusion of *Sambucus nigra* L. blossom in foods and beverages, as well its use in food supplements [15,16].

There are many studies on the bioactive compounds present in medical herbs; however, regarding the *Sambucus nigra* L. blossoms, the information is relatively insufficient. While the chemical composition of elderberries has been comprehensively studied and a lot of applications in pharmacy, in beverage production are known and food, there is still not enough information on leaves and blossoms from the Bulgarian region with respect to the bioactive compounds content. Given the above, our study focuses on the blossom of *Sambucus nigra* L. from four different regions in Bulgaria /Rhodopes, Plovdiv, Strandzha, and Dobrich/ and creating a profile of the chemical composition using spectrophotometric and HPLC methods to determine antioxidant activity, the content of carbohydrates, amino acids, total phenols and flavonoids.

2. Materials and Methods

2.1. Plant Material

Fresh blossoms of *Sambucus nigra* L. from different regions of Bulgaria (Strandzha, Plovdiv, the Rhodope Mountain and Dobrich region) were selected for this study. The blossom clusters of *Sambucus nigra* L. with attached leaves are harvested by cutting when the tree is in full bloom [17]. The intensive flowering period is in May–July, depending on the altitude of the region. The identification of plant material was performed in the Department of Plant and Fungal Diversity and Resources, Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences (IBER-BAS) in Sofia. The department's herbarium is the most representative source of information on the biological diversity of the flora of the Balkans and Bulgaria. It is registered in the Index Herbariorum as an internationally recognized Herbarium with the acronym SOM (Herbarium of Vascular Plants).

2.2. Methods

2.2.1. Drying

Fresh blossoms of *Sambucus nigra* L., intended for the study, were dried in a thin layer in the shade and turned periodically to a constant mass of the sample. The dried samples were homogenized, ground, packed in canvas bags, and stored at 18–20 °C, in the absence of light.

2.2.2. Determination of Antioxidant Activity (AOA)

The samples were extracted with water in a solid to solvent ratio (1:10 w/v) using an ultrasonic bath operating at 40 kHz at 40 °C for 20 min. The antioxidant activity of the extracts was evaluated by four methods based on different mechanisms.

DPPH Assay

The method provides information on the ability to scavenge the radical DPPH (1,1-Diphenyl-2-picrylhydrazyl), based on the mechanisms of mixed transfer of the hydrogen atom (HAT) and single electron transfer. The analyzed sample (0.15 mL) was mixed with 2.85 mL freshly made 0.1 mM DPPH solution (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) in methanol (Merck KGaA, Darmstadt, Germany). The sample was incubated for 15 min at 37 °C in the dark. The reduction in the absorbance at 517 nm was measured by spectrophotometer a VIS spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, the United Kingdom) in comparison to control sample containing methanol and the % inhibition was calculated [18]. A standard curve with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) at a concentration of 0.005–1.0 mM was generated. The results of the assay are presented as millimoles of Trolox equivalents (mM TE)/g dry weight sample.

FRAP Assay

The antioxidant power of ferric ion reduction is based on the mechanism of single electronic transfer. The sample was treated according to [19], with minor modifications. FRAP reagent was prepared by mixing 100 mL of 0.3 M acetate buffer (pH 3.6) (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany), 10 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl (Merck KGaA, Darmstadt, Germany) and 10 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Merck KGaA, Darmstadt, Germany) in distilled water. The reaction was carried out by mixing 3.0 mL of FRAP reagent with 0.1 mL of the studied extract. The reaction time was 10 min at 37 °C in the dark and the absorbance was measured at a wavelength of 593 nm by a VIS spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, the United Kingdom) versus a control sample prepared with methanol (Merck KGaA, Darmstadt, Germany). The antioxidant activity is expressed in mM Trolox® equivalents (TE) per g dry weight.

ABTS Assay

The ABTS radical was prepared by mixing in equimolar amounts ABTS (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) (7 mM in distilled water) and potassium persulphate (2.45 mM in water), which were then left for 16 h in the dark. Prior to the experiment, 2 mL of the solution was mixed with 60 mL methanol (Merck KGaA, Darmstadt, Germany) to obtain a final adsorption of 1.0 ÷ 1.1 at 734 nm by a VIS spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, the United Kingdom). For the analysis, 0.15 mL of the studied sample was mixed with 2.85 mL of freshly made ABTS radical solution. The reaction mixture was incubated in the dark for 15 min at 37 °C. The adsorption reduction was determined spectrophotometrically at a wavelength of 734 nm [20].

CUPRAC Assay

The reaction was initiated by mixing 1 mL of 10 mM $CuCl_2 \times 2H_2O$, 1 mL Neocuproin (7.5 mM in methanol) (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany), 1 mL 0.1 M

ammonium acetate buffer (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany), pH 7, 0.1 mL of tested sample and 1 mL of distilled water. The reaction mixture was incubated for 20 min at 50 °C in the dark. After cooling the mixture, the absorbance was measured at 450 nm using a VIS spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, the United Kingdom) [20]. The results of the assay are presented as millimoles of Trolox equivalents (mM TE)/g dry weight sample.

2.2.3. Determination of Phenolic Content

The total phenolic content in the tested samples was determined by the method of Folin–Ciocalteu [21]. Aqueous extract (0.2 mL) was added to 1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) diluted five times and then 0.8 mL of 7.5% sodium carbonate solution was added. After 20 min in the dark, the absorption was measured at a wavelength of 765 nm by a UV/VIS spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, the United Kingdom) against a blank prepared with distilled water (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany). The concentration of total phenolic content is expressed as milligrams of gallic acid equivalent—mg GAE/g dry weight. The calibration curve is linear in the range of 0.02–0.10 mg gallic acid (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) used as a standard [19].

2.2.4. Determination of Total Flavonoids

Measurement of flavonoid concentration is based on the method described by [22], and the results are expressed as quercetin equivalents. The plant material was extracted with distilled water in a solid to solvent ratio (1:10 w/v). The extraction was performed in duplicate for 20 min at 45 °C in a 35 kHz ultrasonic bath (Isolab Laborgeräte GmbH, Eschau, Germany). The sample was filtered through filter paper and 1 mL of the solution was added to test tubes containing 0.1 mL of 10% aluminum nitrate (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany), 0.1 mL of 1 M potassium acetate (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) and 3.8 mL of 95% ethanol (Merck KGaA, Darmstadt, Germany). After 40 min at room temperature, the absorbance was determined at 415 nm using a VIS spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, the United Kingdom). The results were expressed as mg quercetin equivalent (QE)/g dry sample.

2.2.5. Determination of Carbohydrate Content (HPLC)

The dried *Sambucus nigra* L. flowers and leaves were extracted with distilled water (solid to liquid ratio 1:5 (w/v)) in an ultrasonic bath (Isolab Laborgeräte GmbH, Eschau, Germany) with a frequency of 40 kHz and 60 W power at 50 °C in duplicate. The samples were filtered through a 0.45 µm syringe filter, PTFE45/25 mm (Isolab Laborgeräte GmbH, Eschau, Germany), before injection. The contents of glucose, fructose and sucrose were determined using a Shimadzu HPLC, coupled with an LC-20 ADpump, and a Shimadzu RID-10A refractive index detector (RID). The separation was performed on a Shodex® Sugar SP0810 (300 mm × 8.0 mm i.d.) column coupled with a Shodex SP-Gguard column (5 µm, 6 mm × 50 mm) (Shodex Co., Tokyo, Japan) operating at 85 °C. The mobile phase was ultrapurified water (water purification system Adrona B30 Integrity + HPLC, Riga, Latvia) with a flow rate of 0.5 mL/min. The injection volume was 20 µL [23]. The results were expressed as g/100 g dry weight.

2.2.6. Sweetness Index and Total Sweetness Index

The Sweetness Index (SI) and Total Sweetness Index (TSI) were calculated to determine the sweetness perception. The calculations were performed according to Magwaza and Opara [24], as follows:

$$SI = (1.00 \times [\text{glucose}]) + (2.30 \times [\text{fructose}]) + (1.35 \times [\text{sucrose}])$$

$$TSI = (1.00 \times [\text{sucrose}]) + (0.76 \times [\text{glucose}]) + (1.50 \times [\text{fructose}])$$

2.2.7. Amino Acid Analysis

Dried *Sambucus nigra* L. flowers were subjected to acid hydrolysis using 6N HCl for 24 h at 105 °C. An aliquot of the hydrolysate was derivatized using an AccQ-Fluor reagent kit (Waters, Milford, MA, USA). The derivate was separated on an RP AccQ-Tag™ silica-bonded amino acid column C18, 3.9 mm × 150 mm (Waters, Milford, MA, USA) conditioned at 37 °C using an ELITE LaChrom HPLCsystem (VWR™ Hitachi, Tokyo, Japan). A sample of 20 µL was injected and the elution of the amino acids was performed by a gradient system: eluent A, buffer WAT052890 (Waters, Milford, MA, USA) and eluent B, 60% acetonitrile (Sigma-Aldrich, Merck, Darmstadt, Germany) with a constant flow rate of 1.0 mL/min. The amino acids were detected using a diode array detector (DAD) at 254 nm. The amino acid peaks were then analyzed using EZChromElite™ software and were calculated based on the amino acid standard calibration curve (amino acid standard H, Thermo Fisher Scientific, Waltham, MA, USA). The results are expressed as mg AA/g dry weight (dw) [25].

2.2.8. Mathematical Processing of Results

Five samples of *Sambucus nigra* L. were collected in the area at the indicated geographical point. Five parallel measurements were made for each of the studied parameters. The data on phenolic content, antioxidant activity, sugar content and amino acids were processed to obtain the mean value and standard deviation of the mean value (SD). The analysis of dispersion was used to compare the mean values with a significance level of $p < 0.05$. A one-way analysis of variance and a subsequent Duncan test for multidirectional comparisons based on the investigated parameters were performed for elderberry from different regions of Bulgaria. Statistical analysis was performed using the IBM SPSS Statstic 26, computer program, US.

3. Results and Discussion

3.1. Characteristics of Sambucus nigra L.

Identification of Plant Material

Fresh raw material of *Sambucus nigra* L. from four regions of Bulgaria: Western Rhodopes, Velingrad from the area of Golyam Beglik–Karatepe–Sutka dam (Figure 1a); Plovdiv municipality, village of Staro Zhelezare (Figure 1b); Gramatikovo village, Strandzha region (Figure 1c); Dobrich region (Figure 1d) was studied for authenticity. For simplicity, in the text, those regions are cited as follows: (a) Rhodopes region, (b) Plovdiv region, (c) Strandzha region and (d) Dobrich region.

The identification of plant material makes it possible to draw conclusions about the species, genera, family and evolutionary development of a plant species. According to the requirements of IBEI-BAS, the herbaria of *Sambucus nigra* L. from four regions of Bulgaria were prepared to determine the botanical identity. The presence of morphologically similar species determines the necessity of authenticity evaluation. The locality was described in detail and the geographical coordinates were determined (Table 2).

As can be seen from the photos of different herbaria, there are differences in the morphology of the plants *Sambucus nigra* L. The Elder from the Strandzha region is characterized by the smallest leaves, whereas the Elder from the Rhodopes region has the largest flowers. The plants from the Plovdiv and Dobrich regions have comparable leaf sizes; however, the elder flowers from the Dobrich region are slightly larger than those from the Plovdiv region. It can be concluded that, based on the performed identification, plant species were established, and the identification numbers of each specimen were determined.

Table 3 presents the average carbohydrate composition of the blossoms and leaves of *Sambucus nigra* L. Three sugars were detected in the blossoms and leaves of *Sambucus nigra* L. In leaves, glucose and fructose were the dominating sugars, as glucose presents 49% of the total sugar content. In blossoms, the most abundant sugar was glucose, which occupied 58% of the total sugars in the samples. Our findings in the current research confirmed the reported data [26] for the highest glucose content (as glucose represented from 60%

to 85% total analyzed sugars in elderberry flower). Contrary to the fructose to glucose ratio (1:1) determined in elderberry fruits [27], the glucose/fructose ratio was 1.92 for blossoms and 1.18 for leaves (Table 3). Glucose/fructose ratio coincided with data for peaches [28] and was lower than the glucose-fructose ratio in strawberry and blueberry leaves (1.57–1.65) [29]. This can be explained by the fact that glucose is an essential sugar in plant metabolism, not just for fruit ripening, but also for some other structural, nuclear and biochemical processes in plants (signaling, growth, development and respiration).

(**a**) (**b**)

(**c**) (**d**)

Figure 1. *Sambucus nigra* L. from different regions of Bulgaria. (**a**) Western Rhodopes, Velin-grad, Golyam Beglik–Karatepe–Sutka; (**b**) Staro Zhelezare village, Plovdiv region; (**c**) Gramatikovo village, Strandzha; (**d**) Dobrich region.

It is known that the reduction of sucrose content is extremely important during the elaboration of new food compositions (the World Health Organization recommends limiting added sugars to 5–10% of daily calorie consumption), and very often the aim is to increase the content of monosaccharides (glucose and fructose). Glucose and fructose have the same calories per gram (4 kcal). Glucose is used immediately to generate energy for the body or to be converted into glycogen, which will be stored in the muscles or liver for future use. Similar to glucose, fructose is absorbed directly into the blood across the small intestine. In contrast to glucose, fructose gradually raises blood sugar levels and has an immediate effect on insulin levels.

Table 2. Necessary data for the identification of the studied plant species.

Plant Material/Herbs/, Identification Number	Location			Date
	Locality	Geographical Coordinates	Altitude	
Sambucus nigra L. SOM 1406 n (number of samples) n = 5	Bulgaria, Western Rhodopes, Velingrad, Golyam Beglik-Karatepe-Sutka	35TKG5111032486 Lat. 41.80444 Lon. 24.159444	UTM/MGRS KG53 1271 m	06.07.2021
Sambucus nigra L. SOM 1407 n = 5	Bulgaria, Plovdiv, Staro Zhelezare village	35TKH 54468 37565 Lat. 42. 750555 Lon. 24.000004	UTM/MGRS KH53 294 m	06.05.2021
Sambucus nigra L. SOM 1405 n = 5	Bulgaria, Gramatikovo village, Strandzha region	35TNG0009261486 Lat. 42.104 7222 Lon. 27.001111	UTM/MGRS NG06 295 m	18.06.2021
Sambucus nigra L. SOM 1404 n = 5	Bulgaria, Dobrich region	35TPJ03240 39007 Lat. 43. 696111 Lon. 28.28111	UTM/MGRS PJ03 205 m	09.05.2021

Table 3. Carbohydrate content in samples of *Sambucus nigra* L.

Sample	Leaves	Blossoms
Sucrose, g/100 g	0.55 ± 0.05^{a}	0.26 ± 0.03^{b}
Glucose, g/100 g	3.19 ± 0.02^{a}	1.50 ± 0.05^{b}
Fructose, g/100 g	2.70 ± 0.06^{a}	0.79 ± 0.05^{b}
Total sugars	6.44 ± 0.06^{a}	2.55 ± 0.04^{b}
Sucrose/Glucose	0.17	0.17
Glucose/Fructose	1.18	1.92
Sweetness index	10.1	3.7
Total sweetness index	7.0	2.6

Means in a column with a common superscript letter (a,b) differ ($p < 0.05$) as analyzed by one-way ANOVA.

As shown in Table 3, the flowers and leaves of *Sambucus nigra* L. contain monosaccharides glucose and fructose, and the glucose values are approximately 5.8 times higher than sucrose values in both samples. The leaves have a higher carbohydrate content in comparison to blossoms (6.44% versus 2.55%, respectively). The content of glucose and fructose in the blossoms of *Sambucus nigra* L. in Bulgaria was found to be lower than that established in other European countries [30]. However, the sucrose content is similar to that published in [30]. In our study, sugar content in leaves and flowers decreased in the following order: glucose > fructose > sucrose (Table 3). A similar tendency was found in elderberry fruits, while in elderflowers, it was the opposite [31]. The detected values of sucrose were lower than the reported values for Portuguese elderberry varieties [30].

The lower sucrose content had an impact on the sweet perception of analyzed blossoms and leaves of *Sambucus nigra* L. Leaves demonstrated twice the sweetness and total sweetness indices than blossoms (Table 3). Sweetness indices were 10.1 and 3.7 for the leaves and blossoms of *Sambucus nigra* L., respectively. The values were closer to published values for the sweetness index of strawberry [32].

The distribution of amino acids in g/100 g of the raw materials of *Sambucus nigra* L. is presented in Table 4. The average results for samples of the regions used are presented, as the differences are within the experimental error of the method (±3%).

There are proven statistical differences for elderberry blossoms from the different Bulgarian regions of the respective studied characteristic, at $p < 0.05$. Values for p are not given in the table, where the samples from the respective regions are indistinguishable by that parameter.

Table 4. Amino acid composition of *Sambucus nigra* L. blossoms from different regions in Bulgaria.

Amino Acids, g/100 g	Rhodopes Region	Plovdiv Region	Strandza Region	Dobrich Region	*p* Value
Alanine	0.030 ± 1.10^{-3}	0.030 ± 1.10^{-3}	0.030 ± 1.10^{-3}	0.030 ± 1.10^{-3}	-
Arginine	$0.051 \pm 2.10^{-3\ a}$	$0.049 \pm 2.10^{-3\ ab}$	$0.049 \pm 2.10^{-3\ ab}$	$0.048 \pm 2.10^{-3\ b}$	0.028
Aspartic acid	$0.063 \pm 5.10^{-3\ a}$	$0.058 \pm 4.10^{-3\ b}$	$0.059 \pm 4.10^{-3\ b}$	$0.059 \pm 4.10^{-3\ b}$	0.000
Valine	$0.030 \pm 1.10^{-3\ a}$	$0.029 \pm 2.10^{-3\ a}$	$0.030 \pm 1.10^{-3\ a}$	$0.028 \pm 2.10^{-3\ b}$	0.003
Glycine	$0.042 \pm 5.10^{-3\ a}$	$0.041 \pm 5.10^{-3\ a}$	$0.042 \pm 5.10^{-3\ a}$	$0.038 \pm 5.10^{-3\ c}$	0.000
Glutamine	$0.113 \pm 1.10^{-3\ a}$	$0.098 \pm 1.10^{-3\ b}$	$0.100 \pm 1.10^{-3\ b}$	$0.098 \pm 1.10^{-3\ b}$	0.000
Isoleucine	$0.030 \pm 2.10^{-3\ a}$	$0.029 \pm 2.10^{-3\ ab}$	$0.030 \pm 1.10^{-3\ a}$	$0.029 \pm 2.10^{-3\ b}$	0.030
Leucine	$0.062 \pm 7.10^{-3\ a}$	$0.060 \pm 5.10^{-3\ b}$	$0.060 \pm 5.10^{-3\ b}$	$0.059 \pm 4.10^{-3\ c}$	0.021
Lusine	$0.031 \pm 2.10^{-3\ a}$	$0.029 \pm 1.10^{-3\ b}$	$0.030 \pm 2.10^{-3\ ab}$	$0.028 \pm 1.10^{-3\ c}$	0.000
Methionine	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	-
Proline	$0.031 \pm 3.10^{-3\ a}$	$0.029 \pm 2.10^{-3\ b}$	$0.030 \pm 3.10^{-3\ b}$	$0.029 \pm 2.10^{-3\ b}$	0.01
Serine	0.030 ± 2.10^{-3}	0.030 ± 2.10^{-3}	0.030 ± 2.10^{-3}	0.030 ± 2.10^{-3}	-
Tyrosine	$0.052 \pm 6.10^{-3\ a}$	$0.049 \pm 5.10^{-3\ b}$	$0.050 \pm 6.10^{-3\ b}$	$0.048 \pm 4.10^{-3\ c}$	0.000
Threonine	$0.031 \pm 3.10^{-3\ a}$	$0.029 \pm 2.10^{-3\ b}$	$0.030 \pm 3.10^{-3\ a}$	$0.030 \pm 3.10^{-3\ a}$	0.013
Tryptophan	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	-
Hydroxyproline	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	0.010 ± 1.10^{-3}	-
Phenylalanine	$0.041 \pm 6.10^{-3\ a}$	$0.039 \pm 3.10^{-3\ bc}$	$0.040 \pm 5.10^{-3\ ab}$	$0.038 \pm 4.10^{-3\ c}$	0.020
Histidine	0.020 ± 1.10^{-3}	0.020 ± 1.10^{-3}	0.020 ± 1.10^{-3}	0.020 ± 1.10^{-3}	-
Gystine	0.020 ± 1.10^{-3}	0.020 ± 1.10^{-3}	0.020 ± 1.10^{-3}	0.020 ± 1.10^{-3}	-

Means in a row with a common superscript letter (a–c) differ ($p < 0.05$) as analyzed by one-way ANOVA.

As a result of the Duncan test for most of the amino acids, statistical differences were proved for the blossoms of elderberry from different regions of Bulgaria, except for histidine, gystine, hydroxyproline, tryptophan, methionine and alanine.

It is noteworthy that aspartic, leucine and glutamic acids exhibit the highest content (0.06 ÷ 0.1 g/100 g). It is known that these two amino acids make the greatest contribution to the formation of taste. In addition, Elder contains the full range of eight essential amino acids with leucine, methionine, phenylalanine, valine and lysine and semi-essential arginine, reaching significant levels (between 0.03 g/100 g and 0.05 g/100 g), which determines the beneficial health effect. Vulić et al. defined the amino acid composition of elderberry fruit [33]. The samples collected from mountain regions (Rhodopes and Strandzha) contained the highest level of essential amino acids in a comparison to the samples collected from the valleys (Plovdiv and Dobrich region).

Antioxidant activity was evaluated by four different methods—DPPH; ABTS; FRAP; and CUPRAC assays. The results in mM TE/g dry biomass are shown in Table 5.

Table 5. Antioxidant activity in samples of *Sambucus nigra* L. blossoms from different regions in Bulgaria, mM TE/g dry weight sample (mean ± SD, n = 5).

Sample	DPPH	ABTS	FRAP	CUPRAC
Rhodopes region	236.5 ± 5^{a}	324.5 ± 5^{a}	193.0 ± 2^{a}	748.7 ± 12^{a}
Plovdiv region	160.3 ± 3^{b}	249.8 ± 6^{b}	131.7 ± 2^{b}	554.4 ± 11^{b}
Strandzha region	153.2 ± 5^{b}	240.0 ± 6^{c}	124.4 ± 2^{c}	540.4 ± 23^{b}
Dobrich region	135.1 ± 7^{c}	199.1 ± 7^{d}	101.7 ± 2^{d}	365.1 ± 25^{c}

Means in a column with a common superscript letter (a–d) differ ($p < 0.05$) as analyzed by one-way ANOVA.

The highest antioxidant activity (AOA) was found in *Sambucus nigra* L. blossoms from the region of Rhodopes, irrespective of the assay method used, followed by elder blossoms from the regions of Plovdiv and Strandzha. The lowest values were obtained for the blossoms of *Sambucus nigra* L. from the Dobrich region. Therefore, the AOA of the studied samples may depend on the soil and climate. The AOA according to ABTS assay of the samples of dry blossoms from the Dobrich region was in the range (167–212.74) mM Trolox/g extract, reported also for *Sambucus nigra* L. blossoms from other regions of Europe [34]. The antioxidant activity of elderberry blossoms evaluated by the DPPH method was higher than reported values [35].

A similar trend was also found for the content of biologically active compounds (polyphenols and flavonoids—Table 6). The total phenolic content of *Sambucus nigra* L. blossoms samples from the Dobrich and Plovdiv regions were similar to those published by Dżugan and Viapiana [9,36]. For the blossom samples collected from mountainous regions—Western Rhodopes and Strandzha, it was higher than the published values for samples from other regions of Europe and the world. Moreover, the elderberry blossom in our study demonstrated higher values than those reported for Polish samples (25.34 ± 5.41 mg GAE/g) [36]. The latter may be explained by the climatic conditions and the environment in which the particular plant species grows. A similar conclusion was also reported in another study [37]. According to the authors, the total phenolic content of blossom samples of cultivated *Sambucus nigra* L. is higher than that of the wild species.

Table 6. Total polyphenolic and flavonoid content in samples of *Sambucus nigra* L. from different regions in Bulgaria.

Sample	Total Polyphenols, mg GAE/g Dry Biomass	Total Flavonoids, mg QE/g Dry Biomass
Rhodopes region	49.2 ± 1.0 ^{a}	18.6 ± 0.5 ^{a}
Plovdiv region	36.8 ± 0.5 ^{b}	12.5 ± 0.5 ^{b}
Strandzha region	39.8 ± 2.1 ^{c}	12.4 ± 0.5 ^{b}
Dobrich region	29.3 ± 1.0 ^{d}	6.4 ± 0.5 ^{c}

Means in a column with a common superscript letter (a–d) differ ($p < 0.05$) as analyzed by one-way ANOVA.

A correlation was found between the total phenolic content and antioxidant activity using the four methods. Similar correlations were also reported in [38]. The results for the established regression models are presented in Table 7.

Table 7. Correlation dependences between TPC and antioxidant activity for blossoms of *Sambucus nigra L.* from Bulgaria.

Correlation Dependence	R^2
DPPH = 5.05 TPC + 24.66	0.86
ABTS = 6.15 TPC + 14.84	0.94
FRAP = 4.51 TPC + 37.2	0.90
CUPRAC = 18.73 TPC + 174.26	0.97

The relationships between AOA by CUPRAC and the ABTS method for determining the antioxidant activity and total phenolic content are characterized with highest correlation coefficient.

4. Conclusions

The following conclusions can be drawn on the basis of the obtained results:

– The blossoms of *Sambucus nigra* L from the Rhodope region have the highest antioxidant activity, total phenol content (49.2 ± 1 mgGAE/g) and total flavonoid content (18.6 ± 0.5 mgQE/g)]. The blossoms of *Sambucus nigra* L from the Dobrich region have the lowest antioxidant activity, total phenols and flavonoid content. Probably,

the higher altitude and lower temperature in mountains could influence the accumulation of secondary metabolites in blossoms of *Sambucus nigra* L., which improves the antioxidant potential of the samples.
- The predominant amino acids in all blossoms are glutamic acid, leucine and aspartic acid. The samples collected from mountain regions (Rhodopes and Strandzha) contained the highest level of essential amino acids in comparison to the samples collected from the valleys (Plovdiv and Dobrich region).
- For the first time, the sweetness index for taste perception of elderberry blossoms was evaluated. The concentrations of sugars in the leaves are higher than those in the blossoms. Both parts contained more glucose and fructose than sucrose. The leaves showed twice the indices of sweetness and overall sweetness of the blossoms.

The obtained results were valuable for the nutritional potential of elderberry blossoms as edible flowers with antioxidant potential.

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The Application of Pomegranate, Sugar Apple, and Eggplant Peel Extracts Suppresses *Aspergillus flavus* Growth and Aflatoxin B1 Biosynthesis Pathway

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Abstract: Even though the green revolution was a significant turning point in agriculture, it was also marked by the widespread use of chemical pesticides, which prompted severe concerns about their influence on human and environmental health. As a result, the demand for healthier and more environmentally friendly alternatives to control plant diseases and avoid food spoilage is intensifying. Among the proposed alternatives, food by-product extracts, especially from the most consumed fruits in Egypt, eggplant, sugar apple, and pomegranate peel wastes, were largely ignored. Hence, we chose them to evaluate their antifungal and antiaflatoxigenic activities against maize fungus, *Aspergillus flavus*. All the extracts exhibited multiple degrees of antifungal growth and aflatoxin B1 (AFB1) inhibitory activities (35.52% to 91.18%) in broth media. Additionally, diethyl ether 50% eggplant, ethanol 75% sugar apple, and diethyl ether 25% pomegranate extracts exhibited the highest AFB1 inhibition, of 96.11%, 94.85%, and 78.83%, respectively, after one month of treated-maize storage. At the same time, Topsin fungicide demonstrated an AFB1 inhibition ratio of 72.95%. The relative transcriptional levels of three structural and two regulatory genes, aflD, aflP, aflQ, aflR, and aflS, were downregulated compared to the infected control. The phenolic content (116.88 mg GAEs/g DW) was highest in the 25% diethyl ether pomegranate peel extract, while the antioxidant activity was highest in the 75% ethanol sugar apple extract (94.02 µg/mL). The most abundant active compounds were found in the GC-MS analysis of the fruit peel extracts: α-kaurene, α-fenchene, p-allylphenol, octadecanoic acid, 3,5-dihydroxy phenol, hexestrol, xanthinin, and linoleic acid. Finally, the three fruit peel waste extracts could be a prospective source of friendly ecological compounds that act as environmentally safer and more protective alternatives to inhibit AFB1 production in maize storage.

Keywords: *Aspergillus flavus*; maize; peel extracts; AFB1; GC-MS; qRT-PCR

1. Introduction

Mycotoxins, i.e., aflatoxins, are a type of fungal polyketide secondary metabolite that are produced mostly by *Aspergillus*, including *Aspergillus flavus* [1,2]. Currently, there are 18 types of aflatoxin produced by *Aspergillus* spp., of which the four principal kinds are Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) [3]. AFB1 is the most common mycotoxin in nature and is functionally carcinogenic in animal models as well as mammals if the toxicity level exceeds a certain threshold [4]. Aflatoxigenic fungi can cause damage loss in seeds growth, preservation, or viability [5]. Aflatoxin contamination primarily affects dried fruits (such as nuts and peanuts), cereal grains (maizes, etc.), some spices, and oils [6,7]. The primary sources of the world's exposure to aflatoxins are maize and peanuts because of their high consumption [8]. Consequently, solutions for the control of aflatoxigenic *A. flavus* in maize grains and food during storage are in demand worldwide [9]. Chemical treatments can effectively control aflatoxins, but they cannot be used on grains, cereals, or other food materials due to hazardous residues, teratogenicity, carcinogenicity, spermatotoxicity, and hormonal imbalances, as well as the development of resistance microbes against antimicrobial agents [10–12]. Currently, the use of plant-based natural antifungal agents is considered a beneficial and healthy practice in this regard [13,14]. Plant extracts could be employed as antimicrobial agents or for improved food storage and preservation due to their high activity, the simplicity of their production and utilization, their reliability, and their biocompatibility [15,16].

The release of large quantities of agro-by-product wastes such as peels and seed husks is one of the biggest problems facing society, as they are grave threats to the environment [17,18]. As a result, researchers continue to assess into the possibility of reusing these wastes. Such wastes encompass a wide range of compositions, including high levels of proteins, carbohydrates, and minerals. It was reported that many fruit peels offer a range of biological and medicinal properties and are known to contain them [19,20]. Pomegranate peels, lemon peels, and green walnut husks have been reported to be effective natural antimicrobials in various investigations [21–23]. Pomegranate peel extracts are high in functional molecules, such as flavones, phenylpropanoids, and alkaloids, which feature potent antioxidant properties [24,25]. Many investigators have reported the significant antifungal activity of pomegranate and eggplant peel extracts against many phytopathogens [26–29]. Linoleic acid is known to feature antifungal activity in larger plants as a substrate for producing a series of trihydroxy oxylipins [30]. The growth and biomass production of *Rhizoctonia solani* was reduced by 74% and *Pythium ultimum* by 65% when 1000 µM linolenic acid and allylphenol were applied together [31]. Several studies have reported that such natural compounds, including essential oils or extracts such as monoterpenoids and sesquiterpenes could suppress *A. flavus* growth and AFB1 formation by downregulating the transcription of genes involved in AFB1 synthesis [32,33].

Generally, AFB1 is produced from a complicated biosynthetic pathway, including at least 28 enzymatic steps. The structural genes encoding these enzymes are grouped in one gene cluster while two cluster-specific regulators, aflR and aflS, mainly regulate their expression [34,35]. It was reported that the decrease in the transcription levels of aflatoxin genes was associated with a reduction in AFB1 production. The ability of many plant-derived substances to stop AFB1 production or inhibit its expression has been observed [36–38]. Several investigations have shown that various doses of different plant extracts suppress the expression of 25 of the 27 studied genes in the AFB1 biosynthesis pathway [39,40]. The purpose of this study was to evaluate the effectiveness of different extracts of pomegranate, sugar apple, and eggplant peels to inhibit *A. flavus* growth; to test the ability of the peel extracts to suppress the expression of AFB1 biosynthesis genes in maize grains compared with Topsin fungicide; and to identify the different bioactive compounds of the best extracts using the GC-MS analysis technique.

2. Materials and Methods

2.1. Fungus Isolation and Identification

Aspergillus isolate was isolated from local maize grains, purified, characterized morphologically, and assessed for its ability to produce AFB1. The aflatoxigenic isolate was identified by sequencing the amplified ITS region [41,42].

2.2. Peel Extracts Preparation

The pomegranate (*Punica granatum* L.), sugar apple (*Annona squamosa* L.), and eggplant (*Solanum melongena* L.) edible fruits were purchased from local markets in Alexandria Governorate, Egypt. All the fruits were washed and surface sterilized; the peels obtained, air-dried, and pulverized to a fine powder [43]. Twenty grams of the fine powder for each fruit peel was mixed with 100 mL of each of the four solvents: ethanol, diethyl ether, methanol, and acetone, with three concentrations, of 25%, 50%, and 75% (solvent/water, v/v). The preparations were left overnight on an orbital shaker (Heidolph, Schwabach, Germany) at 200 rpm. All the mixtures were filtered using Whatman No. 1 and stored in a refrigerator (at 5 °C) until further use.

2.3. Total Phenolics Content

The Folin–Ciocalteau reagent (FCR) assay (Sigma-Aldrich, Taufkirchen, Germany) [44], was used to determine the total polyphenols content (TPC), with slight modifications. A total of 0.1 mg/mL of extract was dissolved in distilled water. Next, 0.5 mL FCR (1 mol/L) and 1.5 mL of sodium carbonate (10% w/v) were added to 0.5 mL of each extract. The final mixture was kept for 30 min in the dark, and the absorbance values at a wavelength (λ) = 725 nm were measured. The TPC was calculated according to a standard curve using gallic acid prepared in methanol with 12.5, 25, 50, 75, and 100 µg/mL concentrations. The concentrations of TPC were expressed in milligrams of gallic acid equivalents per gram of dry extract weight (mg GAEs/g DW) [45].

2.4. DPPH Radical Scavenging Ability

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, Taufkirchen, Germany) was used to test antioxidant activity, and the capacities of the extracts to scavenge free radicals were determined as described by Asnaashari et al. [46]. The calculation equation was: $(\mathrm{DPPH})\ \% = [(\mathrm{Ab} - \mathrm{Abs})/\mathrm{Ab}] \times 100$ where Ab is the blank absorbance value and Abs is the sample absorbance value.

2.5. Gas Chromatography-Mass Spectroscopy Analysis

Thermo Scientific ISQ Quadrupole GC-MS with Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a capillary column TG–5MS (30 m × 0.25 mm × 0.25 µm film thickness) was used as previously described [47,48]. The separation conditions were performed as outlined by Okla et al. [49]. For the identification of the different compounds in the fruit peel extract samples, the retention times and mass spectra databases were compared to those of authentic standards.

2.6. Effect of Fruit Peel Extracts on Fungal Biomass and Aflatoxin Production

2.6.1. Fungal Biomass Determination

Fifteen mL of potato dextrose agar (PDA) media was poured into a petri dish, and after the solidification, a 5 mm disc of the aflatoxigenic fungus was placed in the center of the PDA petri dish and incubated for 7 days at 30 °C. In total, 1 mL of each fruit peel extract, previously prepared as described in Section 2.2, was added to 50 mL yeast extract sucrose (YES) broth in a conical flask. Next, a fungus disc was placed in each conical flask and set for 15 days at 30 °C. Each treatment's fungal mat was oven-dried. The wet and dry weights (g) of the fungal mat were recorded in all the treatments, and the filtrates were

maintained at 4 °C for later aflatoxin B1 analysis. The following equation was used to calculate the aflatoxin inhibition (AI) percentage ratio [50]:

$$(\mathrm{AI})\% = \left[\frac{\text{AFB1 control} - \text{AFB1 treatment}}{\text{AFB1 control}}\right] \times 100$$

2.6.2. Maize Storage Experiment

Fifty grams of maize grains were treated with the fruit peel extracts, yielding the most significant results, as in the previous section. The treated grains were placed in sterilized-glass bottles. The fungicide treatment (2.5 mg/mL) was treated with Topsin (Thiophanate methyl, 70% wettable powder, United Phosphorus, Inc., King of Prussia, PA, USA), which was used as a control. Subsequently, each bottle was inoculated with a 5 mm disc of the aflatoxigenic fungus and kept for 30 days at 30 °C. The shape and odor of the maize grains were assessed after the storage period, using the scale developed by Youssef et al. [15]. All of the analyzed grains were crushed and refrigerated at 4 °C until they were used for further aflatoxin studies.

2.6.3. Aflatoxin B1 Extraction

Aflatoxin B1 (AFB1) was extracted by mixing 2 mL of fungal filtrate YES broth medium with chloroform (1:1 v/v). The mixture was centrifuged at 10,000 rpm for 5 min; a total of 2 mL of the bottom layer was transferred to a fresh glass vial. After evaporating under a moderate air stream, the dried chloroform extracts were re-dissolved with 1 mL methanol [51]. To extract the AFB1 from the contaminated maize grains, about 20 g of crushed grains was mixed with 100 mL of methanol and 12 mL of 4% potassium chloride (w/v), according to Hoeltz et al. [52], with some adjustments. The samples were filtered after a spin for 2 min at 10,000 rpm. The filtrate was then added to 100 mL of 10% (w/v) $CuSO_4$, mixed, and filtered. To extract the AFB1, 15 mL of an equal volume of chloroform and distilled water (1:1 v/v) was mixed with the filtrate in the separating funnel; this process was repeated twice. The solvent extracts were collected and evaporated. Before high-performance liquid chromatography (HPLC) analysis, all the samples were filtered into HPLC vials using a 0.2 m syringe filter (Thermo Fisher Scientific, Waltham, MA, USA).

2.6.4. Preparation of AFB1 Standard and HPLC Conditions

To prepare the AFB1 standard (Merck, MO, USA), 1 mg was dissolved in 100 mL of toluene: acetonitrile (9:1, v/v) to obtain a final concentration of 10 µg/L. A working standard solution was prepared with a sample diluent (7% methanol + 92% 0.01 phosphate-buffered saline + 1% dimethylformamide) at concentrations of 5, 2, 1, 0.5, 0.2, and 0 µg/L [53]. The limit of detection and quantification for AFB1 as detected by the UV detector were 0.01 µg/L and 1 µg/L, respectively. Agilent HPLC (Santa Clara, CA, USA) was used to analyze the AFB1 using a Zorbax Eclipse Plus C18 column (4.6 mm 150 mm, 3.5 m) and a UV 365 nm detector. The mobile phase ratios were water, methanol, and acetonitrile (50:40:10, $v/v/v$). The flow rate was 0.8 mL/min, at ambient temperature, and the injection volume was 10 µL, with a concentration of 0.044 mg/mL [51].

2.6.5. RNA Extraction, cDNA Synthesis, and qRT-PCR Assay

The guanidium isothiocyanate technique was used to isolate whole-plant RNA, with certain modifications [54]. As previously described, the reverse transcription procedure was carried out [55,56]. The real-time PCRs (Qiagen Rotor-Gene Q2, Qiagen, Hilden, Germany) were carried out with designated primers targeting the aflatoxin biosynthesis pathway (Table 1). For normalization, the β-tubulin gene was served as an internal reference. As previously indicated, 20 µL SYBR Green qPCR reactions were performed [57,58]. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression levels from the threshold cycle [59].

Table 1. Primer sequences were used in this study.

Gene	Primer Sequences (5′-3′)	Function in the Biosynthesis Pathway of AFB1
β-tubulin	F: CTTGTTGACCAGGTTGTGGAT R: GTCGCAGCCCTCAGCCT	Reference gene
aflD	F: GTCCAAGCAACAGGCCAAGT R: TCGTGCATGTTGGTGATGGT	Norsolorinic acid (NOR) → Averantin (AVN)
aflP	F: GGCCGCCGCTTTGATCTAGG R: ACCACGACCGCCGCC	Sterigmatocystin (ST) → O-methylsterigmatocystin (OMST)
aflQ	F: GTGTCCGCAGTGTCTAGCTT R: GCTCAAAGGTCGCCAGAGTA	OMST → AFB1
aflR	F: CTCAAGGTGCTGGCATGGTA R: CAGCTGCCACTGTTGGTTTC	Regulator gene
aflS	F: CTGCAGCTATATTGCCCACA R: TAAACCCAGGCAGAGTTGGT	Regulator gene

2.7. Statistical Analysis

All the statistical analyses were performed with the CoStat program, version 6.303, and the analysis of variance technique (CoHort software, Monterey, CA, USA). The data from the expression analysis of the aflatoxin biosynthesis genes were expressed as means standard deviation (S.D.), and the values were considered statistically significant when $p \leq 0.05$.

3. Results and Discussion

3.1. Identification of the Aflatoxigenic Fungal Isolate

The aflatoxigenic isolate was found to produce AFB1 at a rate of 25.67 μg/L. The revealed sequence of the ITS region of the aflatoxigenic isolate was submitted to NCBI using a blasting tool given the high similarity with *Aspergillus flavus* fungus [60]. A coded name was given to the isolate as *A. flavus* f2 and GenBank accession no. (# MG202160).

3.2. Fruit Peel Extracts Effect on A. flavus Biomass and AFB1 Production

To evaluate the influence of different fruit peel extracts prepared with varying solvents at three different concentrations on *A. flavus* fungal biomass (dry and wet weight) and AFB1 production, the pomegranate, sugar apple, and eggplant fruit peels were extracted with diethyl ether, acetone, ethanol, and methanol at concentration percentages of 25%, 50%, and 75% (Table 2). In comparison to the control, the 75% ethanol pomegranate peel extract (0.09 and 0.03 g), 25%- methanol sugar apple peel extract (0.12 and 0.01 g), and 75%- methanol eggplant peel extract (1.89 and 0.24 g, respectively) produced the least substantial wet and dry weight values. These results agree with those previously reported for pomegranate peel extract, which has been shown to be intensely active against *A. flavus*, *A. parasiticus*, *A. fumigatus*, *Fusarium proliferatum*, and *F. verticillioides* isolates with minimal inhibitory concentration (MIC) values ranging between 1.25 and 5 mg/mL [61]. Furthermore, similar results were recorded by Oliveira and Furlong [29], according to which eggplant peel phenolic extract inhibited the growth of *A. flavus* after 72 h of incubation with 84.80%. In comparison, Basudan [62]'s findings proved that black and white eggplant peel extracts had no fungal effect against the *A.flavus* strain.

Table 2. *Aspergillus flavus* biomass and AFB1 inhibition ratio (AI%), as affected by applying pomegranate, sugar apple, and eggplant peel extracts.

Solvent Concentration (%)	Control AFB1 Production (µg/L)	Control		Weight of *A. flavus* Mat (g)											
				Pomegranate				Sugar Apple				Eggplant			
		Wet	Dry	Wet	Dry	AFB1 (µg/L)	AI%	Wet	Dry	AFB1 (µg/L)	AI%	Wet	Dry	AFB1 (µg/L)	AI%
Ethanol															
25	23.58	6	0.97	4.56	0.62	2.19	90.70	1.16	0.08	8.52	63.86	6.16	0.88	2.88	87.79
50	24.74	6.97	1.06	4.59	0.44	3.18	87.13	1.19	0.17	5.32	78.49	4.66	0.63	6.79	72.55
75	25.20	3.59	0.62	0.09	0.03	3.69	85.35	0.16	0.05	4.48	82.23	5.60	0.75	2.52	90.60
Acetone															
25	22.49	4.58	0.67	3.78	0.69	2.18	90.32	0.17	0.02	5.97	73.45	3.30	0.62	9.04	59.79
50	20.12	5.05	0.74	4.33	0.93	3.46	82.79	0.14	0.02	6.58	67.29	5.57	0.56	9.60	52.30
75	17.56	4.33	0.73	5.12	1.00	2.57	85.37	0.26	0.01	3.52	79.97	5.19	0.82	10.77	38.66
Methanol															
25	17.00	7.49	0.71	4.34	0.72	6.42	62.26	0.12	0.01	10.50	38.23	5.16	0.57	8.09	52.41
50	13.85	7.16	0.91	4.84	0.74	3.44	75.15	0.32	0.13	8.93	35.52	7.19	0.65	8.69	35.97
75	14.08	5.27	0.62	0.17	0.04	2.60	81.50	5.83	0.75	8.85	37.12	1.89	0.24	4.16	70.43
Diethyl ether															
25	26.44	5.09	0.9	5.83	0.79	2.42	90.87	0.24	0.07	13.71	48.15	5.84	0.80	3.54	86.62
50	24.83	6.08	0.63	4.42	0.85	5.23	78.96	0.19	0.01	5.07	79.59	6.46	0.76	2.19	91.18
75	24.16	7.05	0.73	4.18	0.86	6.86	71.61	2.21	0.39	5.92	75.50	6.31	0.78	2.19	90.95
L.S.D.$_{0.05}$		1.47	0.08	0.47	0.03	0.16		0.02	0.03	0.01		0.49	0.03	0.12	

Furthermore, most of the solvent extracts examined were highly efficient against the formation of AFB1 at various concentrations. The AFB1 inhibition ratio (AI%) ranged from 35.52% to 91.18%. Besides, AFB1 production was less affected by the methanol extracts, with the AI% values ranging from 35.52% to 81.5%. Table 2 shows that pomegranate peel extracts, diethyl ether 25%, ethanol 25%, and acetone 25% produced promising results, with AI values of 90.87%, 90.70%, and 90.32%, respectively. Still, methanol 25% and diethyl ether 75% exhibited the lowest AI values (62.26% and 71.61%, respectively). Table 2 demonstrates that the AI% values of the sugar apple extract with different solvent concentrations differed significantly. In comparison to the AI% values of the other solvents, the highest AI%, of 82.23%, was achieved at 75% ethanol treatment. The least effective ratio was obtained with the 25%, 50%, and 75% methanol sugar apple peel extracts (38.23%, 35.52%, and 37.12%), respectively. Table 2 demonstrates that the highest levels of AFB1 inhibition were obtained with eggplant diethyl ether extract at concentrations of 50% and 75% (91.18% and 90.95%, respectively), while the 50% methanol treatment produced the lowest AI% results (35.97%).

After 72 h of incubation, the pomegranate peel extract at the 1250 µg/mL concentration inhibited AFB1 production by 67% without affecting fungal growth [61]. According to other researchers, extracting antioxidants reduces aflatoxins by absorbing, neutralizing the free radicals, and preventing their proliferation chains, resulting in less dangerous compounds. Furthermore, the efficacy of solvents varies depending on their quantities and the components of a particular plant extract. Sugar apple and eggplant methanol and ethanol peel extracts demonstrated strong antioxidative properties against human infections, according to Bernardo and Sagum [63]; these findings endorse our results. According to Adom et al. [64] and Laddomada et al. [65], the essential antioxidants in maize bran are the phenolic acids, mainly the phenolics covalently bonded with cell wall structural components through ester bonds, which play a defensive role against plant fungal infection.

3.3. Maize Storage Experiment

3.3.1. AFB1 Production

Table 3 demonstrates that 50% diethyl ether eggplant peel extract was the most effective treatment, with the most negligible value of AFB1 (20.72 µg/L) and an AI of 96.11%, followed by 75% ethanol sugar apple peel extract with an AI of 94.85% (27.39 µg/L). Com-

pared to the other treatments, the value of AFB1 in the 25% diethyl ether pomegranate peel extract was 112.64 µg/L. These findings are similar to those of Oliveira and Furlong [20], who found that the presence of 30µg phenol/mL agar eggplant bulb extract can reduce *A. flavus* AFB1 production by 87.80%.

Table 3. AFB1 production (µg/L) and aflatoxin inhibition ratios (AI) after the storage period correspond to peel extract treatments.

Treatments	AFB1 (µg/L)	AI%
Healthy control	0.00	100
Infected control	532	0.00
25%-Diethyl ether pomegranate peel extract	112.64	78.83
75%-Ethanol sugar apple peel extract	27.39	94.85
50%-Diethyl ether eggplant peel extract	20.72	96.11
Topsin fungicide (2.5 mg/mL)	143.92	72.95

3.3.2. Grain Shape and Odor as Affected by Applied Fruit Peel Extract Treatments

Table 4 demonstrates that the tested fruit peel extracts and Topsin fungicide dramatically modified the grains' appearance compared to the control. The eggplant peel extract, followed by the sugar apple and the pomegranate peel extracts, demonstrated outstanding antifungal efficacy and a high-grain appearance compared to the control. The Topsin fungicide treatment of inoculated grains at authorized quantities resulted in rotting, deformation, foul odors, and unapproved grains. Our findings are consistent with those of Gemeda et al. [66] and El-Aziz et al. [67]. They reported similar reductions in *Aspergillus* fungal dry weight after using essential oils. Doum, banana, and licorice peel extracts exhibited similar decreases [15]. The findings were similarly consistent with those of Yazdani et al. [68] and Oliveira and Furlong [29]. They found that some phenolic compounds could reduce aflatoxin production AFB1.

Table 4. Grains' shape and odor correspond to peel extract treatments.

Treatments	Scale	Odor	Shape
Healthy control	5	0	5
Infected control	0	5	0
25% Diethyl ether pomegranate peel extract	4	2	4
75% Ethanol sugar apple peel extract	5	1	5
50% Diethyl ether eggplant peel extract	5	1	5
Topsin fungicide (2.5 mg/mL)	0	5	0

3.4. Transcriptional Levels of AFB1 Biosynthesis Genes

The biosynthesis of aflatoxin compounds, particularly AFB1, is a complicated enzymatic pathway [69]. AFB1 is generated in *A. flavus* from acetyl CoA by a 75 kb cluster of genes that encodes more than 18 enzymatic steps [70,71]. Such pathways are organized by different structural and regulatory genes [72]. These regulatory genes include many genes such as aflR and aflS, while structural genes contain more than 20 genes, such as aflD, aflG, aflH, aflI, aflK, aflM, aflO, aflP, and aflQ [73,74]. In the present study, the influence of the three fruit peel extracts, as well as Topsin fungicide, on the relative expression of two regulatory genes (aflR and aflS) and three structural genes (aflD, aflP, and aflQ) was investigated (Figure 1). The aflD enzyme is required to convert norsolorinic acid to averantin in the early stages of AFB1 biosynthesis. In the late stages of the AFB1 pathway, the aflP and aflQ genes encode enzymes that convert sterigmatocystin to o-methylsterigmatocystin and AFB1, respectively [75,76].

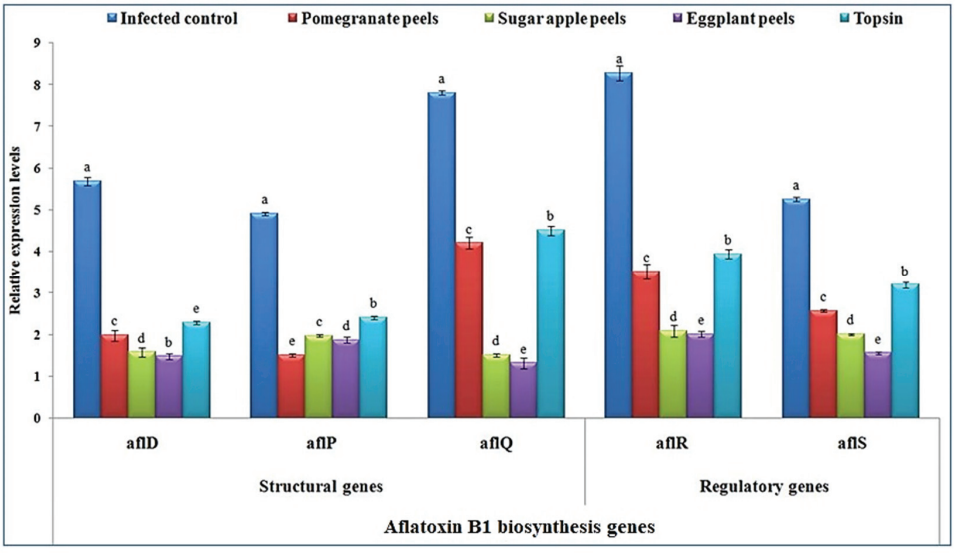

Figure 1. The expression levels of regulatory genes (aflR and aflS) and structural genes (aflD, aflP, and aflQ) of the AFB1 biosynthesis pathway. Columns with the same letters among each gene are not significantly different at $p \leq 0.05$.

The untreated maize grains (infected control) demonstrated the highest relative transcription level of aflD (5.69-fold), followed by the Topsin-treated grains, with a relative expression level 2.29-fold higher than the control (Figure 1). In addition, the acetone 25% pomegranate, diethyl ether 75% sugar apple, and eggplant peel extract treatments showed decreasing transcriptional of aflD, with relative expression levels of 1.98-, 1.58-, and 1.48-fold, respectively (Figure 1). Similarly, the infected control treatment presented the highest relative expressions levels of aflP and aflQ, which were 4.91- and 7.81-fold, respectively, higher than the control (Figure 1). Interestingly, the two-aflP and aflQ genes exhibited the lowest relative expression levels, of 1.51-fold, with acetone 25% pomegranate and diethyl ether 75% eggplant peel extract treatments, respectively (Figure 1). The findings are consistent with those of Mayer et al. [77], who reported that the relative expression level of aflD in wheat experimentally infected with an aflatoxin-producing *A. flavus* was linked with AFB1 production and fungus growth kinetics. As a result, the expression of the aflD, aflP, and aflQ genes can support valuable distinguishing between aflatoxigenic and non-aflatoxigenic *A. flavus* strains [78,79]. Generally, the aflR and aflS are essential regulatory genes for AFB1 synthesis. Many reports assed significant correlations between the transcriptional levels of the two regulatory genes and AFB1 production [15,80]. The results showed the downregulation of two genes, aflR, and aflS, for all treatments compared to infected (untreated) controls. The diethyl ether 75% eggplant peel extract treatment exhibited the lowest aflR and alfS relative expression levels of 2.01- and 1.56-fold, respectively (Figure 1). The untreated infected control exhibited relative expression levels of 8.28- and 5.26-fold for aflR and alfS, respectively (Figure 1). Similar to the structural genes, it was demonstrated that the expression levels of aflS and aflD could be used to distinguish AFB1-producing *Aspergillus* strains from non-producing *Aspergillus* strains [81]. The results indicated that all the tested extracts exert an inhibitory effect on growth and aflatoxin production and could be used as antimycotoxigenic agents against AFB1-producing *Aspergillus* strains.

3.5. Total Phenolics Content of the Fruit Peel Extracts

The TPC was calculated as mg GAEs/g DW for each of the plant materials evaluated. The fruit peel extracts tested presented TPC levels ranging from 30.26 to 116.88 mg of GAEs/g DW (Table 5). The 25% diethyl ether pomegranate peel extract (116.88 mg GAEs/g

DW) featured the greatest TPC. The TPC of the 75% ethanol sugar apple peel extract was 35.09 mg GAEs/g DW, and 50% diethyl ether eggplant peel extract featured the lowest TPC value (30.26 mg GAEs/g DW). It was shown that TPC values in various 70% ethanol pomegranate peel fractions ranged from 78.10 to 200.90 mg GAE/g. Meanwhile, the ethyl acetate fraction featured the highest TPC, whereas the petrol-ether fraction presented the lowest. In the ethyl acetate fraction, gallic acid and ellagic acid were found in the highest quantities. However, in the water fraction, punicalin and punicalagin were found in the highest concentrations [82]. Manochai et al. [83] found that TPC ranged from 33.80 to 140.40 mg GAEs/g in ten Thailand sugar apple peel ethanol extracts, while Petch Pakchong cultivar featured the lowest TPC, of 33.80 mg GAEs/g. In another study, Ji et al. [84] found that the total phenolic content of eggplant peel water extract was more than four times higher than that of eggplant pulp. Malviya et al. [85] reported that the highest value of TPC was detected in a 100% aqueous pomegranate extract. At the same time, the lowest TPC was found in the 70% ethanol extracts. Phenolic contents are the most common secondary metabolites in plants. Their high antioxidant activities and significant impact in preventing oxidative stress-related disorders have drawn increasing attention [86].

Table 5. Total phenolics (TPC) and total antioxidant activity (TAA) of the fruit peel extracts.

Fruit Peel Extract	TPC (mgGAEs/g DW) ± SD	TAA (μg/mL) ± SD
25% Diethyl ether pomegranate	116.88 ± 1.44	86.76 ± 0.22
75% Ethanol sugar apple	35.09 ± 1.79	94.02 ± 0.08
50% Diethyl ether eggplant	30.26 ± 1.76	52.94 ± 0.15

3.6. DPPH Scavenging Ability

DPPH is a method for evaluating the antioxidant potential of extracts and testing the ability of substances to serve as free radical scavengers or hydrogen donors [87]. For DPPH scavenging IC_{50} values, total antioxidant activities (TAA) were evaluated in all the investigated fruit peels. The results revealed that most of the extracted peels examined featured reasonably strong antioxidative activity values (Table 5). The highest TAA was found in the sugar apple peel extract (94.02 μg/mL), followed by 86.76 and 52.94 μg/mL in pomegranate and eggplant peels, respectively. The findings were similar to those of Ji et al. [84], who observed a higher amount of ascorbic acid in eggplant peel (51.88 mg/100 g). Meanwhile, Jayaprakasha and Rao [88]'s results suggested that methanol pomegranate peel extract offered the highest antioxidant activity among all the tested extracts in scavenging or preventive capacity against superoxide anion, hydroxyl, and peroxyl radicals [89]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was found to be the highest for the methanol pomegranate peel extract and the 70% aqueous ethanol extract (79.50; 94.60), respectively [85]. Ten Thailand cultivars of sugar apple peel extracts featured antioxidant activity ranging between 0.42 and 3.06 mg/mL, and the "Nhur Thong" cultivar peel ethanol extract featured the highest antioxidant capacity [83].

3.7. Bioactive Compounds Identified in Fruit Peel Extracts

The GC-MS analysis of the pomegranate, sugar apple, and eggplant peel extracts revealed many bioactive components in each extract. Table 6 presents the main extract compounds with peak area percentages (%) at different retention times (RT). The pomegranate peel extract included a total of 29 components. High relative abundance concentrations were observed in p-allylphenol (20.78%), 3,5 dihydroxy phenol (9.37%), linoleic acid (7.35%), xanthinin (7.05%), sorbitol (5.66%), ethylnorbornane (4.93%), levoglucosenone (4.76%), D-mannose (4.70%), α-himachalene (4.33%), and octadecanoic acid (3.65%). The sugar apple peel extract included a total of 32 components, with a high relative abundance concentration in α-fenchene (11.03%), octadecanoic acid (10.34%), alpha-kaurene (6.33%), hexestrol (5.87%), longipinene (5.83%), methyl isopimarate (5.67%), rhodopin (5.61%), valproic acid (5.04%), (S)-(-)-citronellic acid (4.76%), 4-methylcatechol (4.75%), and 1,16-hexadecanedioic acid (4.20%). As stated previously, the polyphenol content of the pomegranate peel extract

included ellagic and punicalagin compounds, or their derivatives [90]. The eggplant peel extract included a total of 43 components, with a high relative abundance concentration of alpha-kaurene (25.67%), p-allylphenol (8.50%), methyl isopimarate (6.01%), linoleic acid (5.22%), α-fenchene (4.67%), phyllocladene (4.29%), rhodopin (4.19%), octadecanoic acid (3.98%), dimrthoxydurene (3.76%), and (+)-beyerne (3.03%). The most relative abundance of octadecanoic acid, the ester of linoleic acid, which operates as a signaling molecule, was 10.34%, at an RT of 15.70 min.

Table 6. GC-MS phytochemical analysis of pomegranate, sugar apple, and eggplant peels extracts.

Retention Time	Compound	Class	Peak Area (%)		
			Pomegranate	Sugar Apple	Eggplant
3.70	α-Fenchene	Bicyclic monoterpene	-	11.03	4.67
4.90	p-Allylphenol	Phenylpropene	20.78	2.11	8.50
5.10	Valproic acid	Saturated fatty acids	-	5.04	0.79
5.31	Ethylnorbornene	Cyclic hydrocarbons	4.93	1.14	1.68
5.60	Phenylglyoxylic acid	Carboxylic acids	2.26	1.43	0.62
6.20	4-Methylcatechol	Polyphenols	0.76	4.74	0.93
6.34	Sorbitol	Sugar alcohols	5.66	-	-
6.60	7,8-Dihydro-α-ionone	Carotenoids	-	2.79	0.51
6.63	4-Ethylbezaldehyde	Aldehydes	3.07	-	-
6.88	Dimethoxy durene	Alkylbenzene	0.57	0.77	3.76
7.05	Dimethyl caffeic acid	Phenolic acids	1.66	-	-
7.15	Scopoletin	Phenylpropanoids	0.62	-	-
7.45	Farnesol	Sesquiterpenes	0.29	1.87	0.46
7.70	Hexestrol	Nonsteroidal estrogen	0.35	5.87	0.29
7.91	*p*-Cymene	Monoterpenes	1.00	-	0.37
8.04	α-Terpineol	Monoterpenes	-	-	0.59
8.11	Stevioside	Diterpene glycosides	1.29	0.43	0.43
8.27	γ-Terpinene	Monoterpenes	-	3.24	0.32
8.55	Levoglucosenone	Heterocyclic ketones	4.76	-	-
8.59	6-Hydroxyflavone	Flavonoids	0.78	0.39	0.35
8.94	Resveratrol	Polyphenols	-	0.69	0.61
9.49	N-Acetylneuraminic acid	Alpha-keto acid sugars	-	3.01	-
9.76	D-mannose	Carbohydrates	4.70	-	-
9.90	Xanthinin	Sesquiterpene lactones	7.05	3.80	0.53
10.10	3,5-Dihydroxyphenol	Phenoles	9.37	-	-
10.63	3,5,7-Tri-O-methylgalangin	Flavonoids	-	0.50	-
11.00	*p*-Menthone	Monoterpenes	0.96	-	-
11.20	δ-Elemene	Sesquiterpenes	-	-	2.14
11.47	β-lonol	Sesquiterpenes	1.35	-	-
11.67	α-Selinene	Sesquiterpenes	-	-	0.70
12.00	Caryophyllene	Sesquiterpenes	-	-	0.69
12.26	Kaempferol	Flavonoids	-	-	0.26
12.34	5,7,3′,4′-Tetrahydroxflavanone	Flavonoids	-	-	0.54
12.47	β-Patchoulene	Polycyclic hydrocarbons	-	-	1.09
12.55	β-Gurjunene	Sesquiterpenes	-	-	0.67
12.76	γ-Muurolene	Sesquiterpenes	-	-	0.74
13.01	Quercetin 7, 3, 4—Trimethoxy	Flavonoids	1.50	-	-
13.20	α-Himachalene	Sesquiterpenes	4.33	-	-
13.70	Longipinene	Epoxides	-	5.83	0.72
14.95	Apigenin 8-C-glucoside	Flavonoids	-	0.65	-
15.10	4-Hydroxy-2-methoxybenzaldehyde	Methoxyphenols	-	0.19	-
15.42	Methyl 17-methyloctadecanoate	Fatty acid methyl esters	0.67	0.23	-
15.70	Octadecanoic acid	Fatty acids	3.65	10.34	3.98
15.85	Glycitein	Isoflavones	0.74	-	-
15.85	Stearic acid	Fatty acids	-	2.17	0.91

Table 6. *Cont.*

Retention Time	Compound	Class	Peak Area (%)		
			Pomegranate	Sugar Apple	Eggplant
16.19	Phyllocladene	Diterpenoids	-	-	4.29
16.52	Luteolin 6,8-C-diglucoside	Flavonoids	2.03	-	1.42
16.91	(S)-(-)-Citronellic acid	Monoterpenes	-	4.75	-
16.91	Linoleic acid	Polyunsaturated fatty acids	7.35	-	5.22
17.04	5β, 7Bh, 10α-Eudesm-11-en-1α-ol	Sesquiterpenes	0.78	-	
17.05	Quinine	Alkaloids	-	-	0.28
17.10	1,16-Hexadecanedioic acid	Fatty acids	-	4.20	1.40
17.79	Tetrahydroisovelleral	Sesquiterpene dialdehydes	-	-	2.29
18.26	2β-hydroxy-9-oxoverrucosane	Terepnoides	-	-	1.04
18.60	(+)-Beyerene	Diterpenes	-	1.92	3.03
18.70	Abietic acid	Diterpenes	-	-	1.84
19.22	α-Kaurene	Diterpenes	-	6.33	25.67
19.89	Methyl isopimarate	Diterpenes	-	5.67	6.01
21.31	Isosteviol	Diterpenes	-	0.36	2.54
21.62	7α-Hydroxymanool	Diterpenes	-	0.33	1.15
23.08	Sclareol	Diterpene alcohols	-	1.56	1.08
23.65	Zeaxanthin	Carotenoids	2.09	-	-
23.71	Rhodopin	Carotenoids	-	5.61	4.19

It was reported that linolenic acid and p-allylphenol (syn. chavicol) possess antifungal properties [91,92]. They may help to manage plant infections, such as *Botrytis cinerea*, which increases fungal oxygen consumption by 19.58% at 5 ppm and features fungicidal properties against various taxa, including *Alternaria* and *Sclerotinia* species [91,92]. Hydroxychavicol derivative found in betel leaf chloroform extract inhibited *Aspergillus* species with minimum fungicidal concentration (MFC) ranging from 125 to 500 μg/mL employed with broth microdilution method [93]. Similarly, several linoleic acid derivatives have been shown to inhibit the production of AFB1 by various *Aspergillus* species [68].

The second abundant phenolic compound found in the pomegranate peel extract was 3,5-dihydroxy phenol (syn., phloroglucinol). Acylated phloroglucinol is employed as an antifungal agent against *A. flavus* and *A. niger* at a low minimum inhibitory concentration (MIC), 1.0 μg/mL [94]. Flavones, catechins, tannins, and quinines are phenolic compounds that interact with proteins and inactivate them by modifying their structure. Different phenolic compounds, such as dihydrochalcone and chavibetol, were identified from *Piper betle* extract, as reported by Ali et al. [93] and Yazdani et al. [68]. Polyphenolic compounds could halt the *A. flavus* production pathway of AFB1 by reducing norsolorinic acid accumulation, according to Hua et al. [95]. Pomegranate peel extract also contains xanthinin, which acts as a plant growth regulator and features phytotoxicity. It was purified from *Xanthium macrocarpum* fruit and evaluated against *A. fumigates*; it exhibited antifungal activity with MIC > 250 μg/mL. A possible metabolization leading to an unsaturated carbonyl group by eliminating an acetate ion could thus explain its antifungal activity [96]. Furthermore, the pomegranate and eggplant peel extracts contain the energy source linoleic acid, which is crucial for maintaining the membrane fluidity of the epidermis' transdermal water barrier. The biological inhibitory effect of hexestrol was investigated in a study by Inamori et al. [97], who found potent antifungal activity on the growth of plant pathogenic fungi, *Fusarium oxysporum f.* sp. *lycopersici* and *Botrytinia fuckeliana* with MIC 5 and 10 μg/mL, respectively.

Terpenes are the most diverse category of bioactive molecules identified in many plant extracts, with significant antibacterial activities that can be boosted synergistically through the interplay of multiple compounds (from the plant's crude extracts). The biochemical composition of these extracts varies based on the plant species and the plant part used [98]. The compound α-fenchene, also known as (-)-7,7-dimethyl-2-methylene bi-cycle [2.2.1]

heptane, was the most organic compound in sugar apple peel extract considered bicyclic monoterpenoids. The α-kaurene (ent-kaurene) was the most diterpene chemical compound detected in the eggplant peel extract. Low antimicrobial properties of ent-kaurene against *E. coli*, *P. aeruginosa*, *S. aureus*, *C. albicans T. mentagrophytes*, and *T. rubrum* were observed; in addition, no activity was observed against *A. niger* at 30 μg/mL [99,100]. Since monoterpenes, diterpenes, and sesquiterpenes were detected in the three studied fruit peel extracts, they could be effective alone or synergistically at killing fungi or preventing aflatoxin production in media or stored grains. Similarly, Bisht et al. [101] found that *Origanum vulgare* hydro-distilled oil (the main constituent is the oxygenated monoterpene *p*-cymene) strongly inhibited both fungi *A. flavus* and *A. niger*, with the highest inhibition zone of 30 mm. Linalool and citral terpenes are effective against *C. albicans*, and when combined with fluconazole, they produce tremendous synergistic action against a fluconazole-resistant *C. albicans* [102].

Even though eggplant peel extract includes a high amount of polyphenols, terpenoids, and fatty acids, it suppresses *A. flavus* AFB1 production by up to 95%. By contrast, a combined treatment (low dosages of pomegranate peel extract and the azole fungicide prochloraz) resulted in the total prevention of toxin synthesis over 72 h. A qRT-PCR analysis revealed the downregulation of most aflatoxin biosynthetic cluster genes [61]. Youssef et al. [48] reported that phytochemical compounds, such as fatty acids or their esters (octadecanoic acid, n-hexadecanoic acid, and hexadecanoic acid methyl ester), as identified in beetroot extracts, offered potential activity against the mycotoxin produced by *Alternaria alternata*. Overall, these findings suggest that fruit peel extracts could be promising as efficient and sustainable green sources of antioxidants, inhibit aflatoxin production, and potentially become protective grain storage saver applications instead of the chemicals currently used.

4. Conclusions

Among three concentrations of four different extracts of pomegranate, sugar apple, and eggplant peels applied as inhibitors for aflatoxigenic maize fungus *A. flavus*, the diethyl ether 50% eggplant extract displayed the highest AFB1 inhibition ratio (91.18%). After one month of maize grain storage, all the studied peel extracts were effective against AFB1 production, with average inhibition ratios ranging from 78.83% to 96.11% compared to Topsin fungicide (72.95%). The relative levels of aflD, aflP, aflQ, aflR, and aflS expression were considerably down-regulated compared to the untreated maize grains. GC-MS phytochemical analysis of fruit peel extracts suggests that compounds such as; α-kaurene, α-fenchene, *p*-allylphenol, octadecanoic acid, 3,5-dihydroxy phenol, hexestrol, xanthinin, and linoleic acid could provide antioxidant capacity, antifungal properties, and finally suppress aflatoxin production.

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Optical Properties and Antioxidant Activity of Water-Ethanolic Extracts from *Sempervivum tectorum* L. from Bulgaria

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Abstract: *Sempervivum tectorum* L. is an evergreen plant belonging to a large family of the Crassulaceae. The fresh juice of the plant is used as a folk remedy almost exclusively for external purposes. The combination of several instrumental methods—transmission and fluorescence spectroscopy, ICP-MS spectrometry, and assays for the assessment of antioxidant activities were used for the characterization of water-ethanolic extracts from the leaves of *Sempervivum tectorum* L. with different polarities (ethanol content in the extracts varied between 10% and 95%). The highest total phenolic content was found in the 50% ethanol extract—0.84 ± 0.08 mg GAE/mL. Furthermore, this extract also possessed the highest antioxidant potential evaluated by DPPH and FRAP assays—7.02 ± 0.71 mM TE/mL and 6.15 ± 0.25 mM TE/mL, respectively. High correlation coefficients were found between the total phenolic contents and the antioxidant activities of water-ethanolic extracts from *Sempervivum tectorum* L. The same is true for the strong relationship between the phenolic contents and the concentrations of Na and K. Most likely, the bioavailable species of elements such as Fe, Zn, Ca, and Mg are mostly aqueous soluble. For all the studied extracts, the toxic element (As, Cd, Pb, Tl, Hg) contents are very much below the permissible limits for pharmaceutical products. On the contrary, the concentrations of compounds such as β-carotene and chlorophyll increase with the increase in ethanol in the extract. Results from this study may be used for the preliminary prognosis of pharmaceutical applications of extracts from *Sempervivum tectorum* L.

Keywords: *Sempervivum tectorum* L.; antioxidant activity; fluorescence; total phenolic content; heavy metals

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1. Introduction

Egypt, Iran, China, and India have known about and applied the healing properties of some plants for more than 3000 years. Ancient scientists (Hippocrates, Theophrastus, Avicenna, and many others) have described the herbs used in their time. The first records of the use of regional herbs date back to the time of Theophrastus. In his work, "On Medicines", Dioscorides, the most famous pharmacologist of antiquity, describes the herbs used by the Thracians [1]. Even today, it is known that medicinal plants are a valuable source for making medicines. Many of the medicinal plants are the basis for obtaining nutritional supplements to reduce the action of free radicals and reduce oxidative stress in living cells [2–4]. Aromatic and other biologically active substances are extracted from many herbs [5–7].

In recent years, studies have been focused on the concentrations of potentially bioactive compounds in various plants and their antioxidant activities, detoxifying properties, and other essential properties for human health.

Plants offer a wide range of natural antioxidants. Many herbal decoctions and extracts have been widely used in folk medicine for centuries. *Sempervivum tectorum* is a plant known in folk medicine with fleshy, succulent leaves; the juice is used to treat inflammation of the ears, mild burns and wounds on the skin, warts, ulcers, skin rashes, and calluses [8–10]. The potent antioxidant activity and the ability to inhibit lipid peroxidation have been established [11,12], as well as the membrane-stabilizing and protective effects on the liver [13]. Drinking tea prepared from the leaves of the plant is recommended for stomach ulcers [14]. Chromatographically purified fractions of *S. tectorum* L. in which rare natural components have been identified—monosaccharide sedoheptulose and polyalcohol 2-C-methyl-D-erythritol, as well as known organic acids and flavonoids, demonstrate the therapeutic effects of the plant in the healing of wounds [15]. *Sempervivum tectorum* extracts have been used to normalize oxidative stress biomarkers in the blood of rats [16].

Sempervivum tectorum L., although often used in folk medicine, has been poorly studied. The available data in the scientific literature are still few [8,15,17]. Although several pharmacological properties have been reported for the leaves of *Sempervivum tectorum* L., a complete characterization of the extracts is not yet available. Therefore, only the polyphenolic contents and, in part, the antioxidant activities have been studied [16,18].

The present study aims to: (i) determine the optical characteristics, antioxidant activities, and elemental composition of water-ethanolic extracts of *Sempervivum tectorum* L. with different polarities; (ii) elucidate the correlation between antioxidant activities and the contents of bioactive compounds such as polyphenols and beta carotene, and (iii) demonstrate the possible relationship between the essential element contents and polyphenols.

2. Materials and Methods

Plant materials.

The above ground parts of the *Sempervivum tectorum* L (leaves) were sampled randomly from the parks of Sofia in April 2021. Samples were botanically identified by Assoc. Prof. Iliya Slavov (Department of Botany, Faculty of Pharmacy, MU-Varna). *Sempervivum tectorum* L. is a cultivated ornamental plant and was not present in the herbarium until now.

Samples were washed with distilled water, dried at 40 °C to constant weight, and further ground in a coffee grinder.

Preparation of water-ethanolic extracts.

The dried and ground leaves of the plants were treated with different concentrations of ethanol (C_2H_5OH 96%, Sigma-Aldrich, Darmstadt, Germany)—10%, 50%, and 95% *v/v*, respectively. In all cases, the sample to alcohol ratio was 1:10 w/v. The extraction was performed for 48 h at room temperature by constantly shaking the sample at 50 rpm (Digital orbital shaker, SHO-2D, witeg Labortechnik GmbH, Wertheim, Germany). Finally, the resulting extracts were separated by filtration—0.20 μm filters (CHROMAFIL® CA-20/25, Düren, Germany) and kept in glass vessels in dark places.

Elemental composition.

To determine the elements in the water-ethanolic extracts, 10 mL was carefully dried under an IR lamp (until complete alcohol removal) and treated with nitric acid (65%, Suprapur®, Merck, Darmstadt, Germany) for the mineralization of the extracted organic components. The drying of the ethanol extracts and their further digestion was performed at a temperature below 40 °C, ensuring loss free determination of As and Hg. After digestion, the solutions were diluted with doubly distilled water to a final volume of 25 mL.

The quantitative determination of chemical elements was carried out using ICP-MS ("X SERIES 2"—Thermo Scientific, (Thermo Fisher Scientific, Bremen, Germany) under optimal instrumental parameters [19]. Multielement standard solution 5 for ICP (TraceCERT®,

Sigma-Aldrich Production GmbH, Industriestrasse 25, 9471 Buchs, Switzerland, subsidiary of Merck) and standard solutions of Hg and As (TraceCERT®, 1000 mg/L, Merck) were used for the preparation of diluted working standard solutions for the calibration of ICP-MS.

Optical measurements.

The test samples' color coordinates, color parameters, and brightness were measured with a Lovibond PFX 880 (UK) colorimeter in 10 mm cells at room temperature. Using the spectrum in the visible range and the values for the color parameters utilizing a software program developed especially for Lovibond PFX 880 by the manufacturer, chlorophyll and β-carotene were calculated.

The CIELab (1976) colorimetric system characterizing the colors of the extracts was used. In this colorimetric system, the color components a and b characterize the predominance of the red or green component and the yellow or blue component in the respective samples. The parameter L is called luminosity, and the smaller its value, the darker the sample.

The fluorescence of the ethanol extracts was studied by exciting them with light-emitting diodes (LEDs), emitting at 370 nm, 395 nm, 405 nm, 410 nm, 425 nm, 435 nm, and 450 nm. A 90-degree geometry for light detection in a 10 × 10 mm cuvette was used. Samples were studied without any preliminary solution. Fluorescence and scattering spectra were recorded using the fiber optic spectrometer Avantes 2048 with a spectral sensitivity within the 250–1100 nm range.

The resolution of the spectrometer is 8 nm for an input slit of 200 μm. An optical fiber with a diameter of 200 μm is used to bring the light to the probe and to measure the scattered and fluorescent light. A collimator with a lens with an aperture of D = 5 mm is used to collect more light and send it to the receiver. In general, in classical fluorescence spectroscopy, measurements are performed in dilute solutions where the absorbance is below 0.1. At higher optical densities, the fluorescence intensity decreases due to the effect of the internal filter. In this case, frontal-face fluorescence spectroscopy is more suitable for use. In the present study, to measure the fluorescence spectra of extracts without dilution, the cuvette holder is modified as follows: the first probe (optical fiber) is placed directly in the test sample, and the second probe is fixed on top of the drop surface.

Phenolic contents.

Total phenolic contents (TPC) were determined using a Folin-Ciocalteu reagent (Sigma-Aldrich, Germany) [20] with some modifications. Briefly, Folin-Ciocalteu reagent (1 mL) diluted five times was mixed with 0.2 mL sample and 0.8 mL 7.5% Na_2CO_3 (Sigma-Aldrich, Germany). The reaction was performed for 20 min at room temperature in darkness. Then the absorbance was measured at 765 nm by a UV/Vis spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, UK) against a blank, prepared with 70% methanol (Sigma-Aldrich, Germany). The calibration curve was linear in the range of 0.02–0.10 mg gallic acid (Sigma-Aldrich, Germany) and was used as a standard [21].

Antioxidant activities.

Two methods were used to evaluate the antioxidant activities: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical based on mixed hydrogen atom transfer (HAT) and single electron transfer mechanisms and FRAP (ferric reducing antioxidant power) based only on a single electron transfer mechanism.

For the DPPH radical-scavenging ability, the analyzed sample (0.15 mL) was mixed with 2.85 mL freshly prepared 0.1 mM solution of DPPH (Sigma-Aldrich, Germany) in methanol (Merck). The sample was incubated for 15 min at 37 °C in darkness. The reduction of absorbance at 517 nm was measured by spectrophotometer a Vis spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., UK) compared to the blank containing methanol, and the percentage of inhibition was calculated [22]. A standard curve was built with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, Germany) in concentrations of between 0.005 and 1.0 mM.

The Ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain [23] with a slight modification. The reagent was freshly prepared by

mixing 10 parts 0.3 M acetate buffer (pH 3.6) (Sigma-Aldrich, Germany), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) (Fluka) in 40 mM HCl (Merck, Germany) and 1 part 20 mM $FeCl_3.6H_2O$ (Merck, Germany) in distilled H_2O. The reaction was started by mixing 3.0 mL FRAP reagent with 0.1 mL of investigated extract. The reaction time was 10 min at 37 °C in darkness, and the absorbance was measured at 593 nm by a Vis spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., UK) against a blank prepared with methanol (Merck, Germany).

Statistical Analysis.

Data for the contents of pigments, phenols, antioxidant activity, and color characteristics were processed to obtain the mean and standard deviation of the mean (SD). One-way analysis of variance followed by a Student's *t*-test was used to compare the mean values. A value of $p < 0.05$ was considered to be statistically significant.

The linear dependences between the studied parameters were obtained by performing a one-way analysis of variance. To estimate the parameters of the regression model, the least squares method was applied. The coefficient of determination was determined and the adequacy of the model was checked using IBM SPS software.

3. Results

The results for the total phenolic contents and antioxidant activities of the obtained plant extracts are summarized in Table 1. Two methods evaluated the antioxidant activities of extracts: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging ability assay based on mixed hydrogen atom transfer (HAT) and FRAP (ferric reducing antioxidant power) assay based only on a single electron transfer mechanism.

Table 1. Total phenolic content (mg GAE/mL) and in vitro antioxidant activities (mM TE/mL) in extracts.

Sample	TPC, mg GAE/mL	DPPH, mM TE/mL	FRAP, mM TE/mL
10% ethanolic extracts	0.49 ± 0.05	4.06 ± 0.47	3.85 ± 0.30
50% ethanolic extracts	0.84 ± 0.08	7.02 ± 0.71	6.15 ± 0.25
95% ethanolic extracts	0.18 ± 0.04	1.86 ± 0.09	1.56 ± 0.47

The color coordinates were obtained in two different colorimetric systems XYZ and CIELab. The results are presented in Table 2.

Table 2. Color parameters in two different colorimetric systems.

Sample	x ± 2.10^{-4}	y ± 2.10^{-4}	a	b
10% ethanolic extracts	0.3314	0.3381	10.25	0.08
50% ethanolic extracts	0.3426	0.3507	14.87	0.32
95% ethanolic extracts	0.3510	0.3762	18.82	−4.98

The chromaticity and color tone angle were determined by using the data in Table 2. The brightness L decreases and the chromaticity increases when the ethanol concentration is high. The values for the color tone angle of 10% and 50% water-ethanolic extracts are between 88–90 degrees, but the value is −75 degrees for the 95% ethanol concentration.

Transmission spectra for the investigated samples have been obtained (Figure 1). Chlorophyll and β-carotene are computed based on the transmission spectrum of the sample, and the color characteristics are obtained using the specially developed soft wear application Lovibond™ PFX/PFXi Software Upgrade for Chlorophyll and Beta-Carotene. The average results and standard deviations are shown in Table 3.

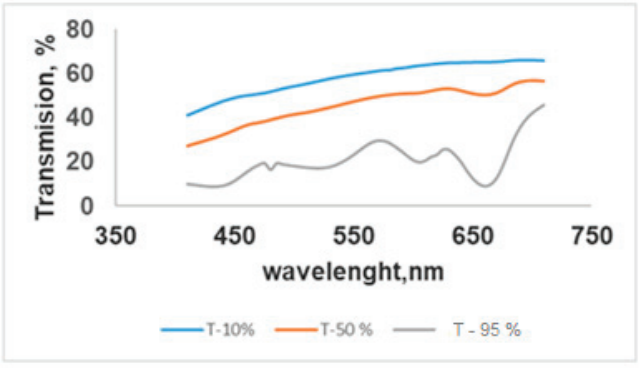


Figure 1. Transmission spectra for ethanol extracts of *Sempervivum tectorum* L.

Table 3. Content of pigments in ethanol extracts.

Sample	Chlorophyll Content, ppm	β-Carotene, ppm
10% ethanolic extracts	<DL *	3.51 ± 0.01
50% ethanolic extracts	0.31 ± 0.01	5.89 ± 0.03
95% ethanolic extracts	4.74 ± 0.03	11.12 ± 0.07

* Detection limit (DL) 0.01 ppm.

The linear dependencies between the color parameters, obtained in Table 2, and the contents of β-carotene from Table 3 were obtained. They are presented in Figure 2.

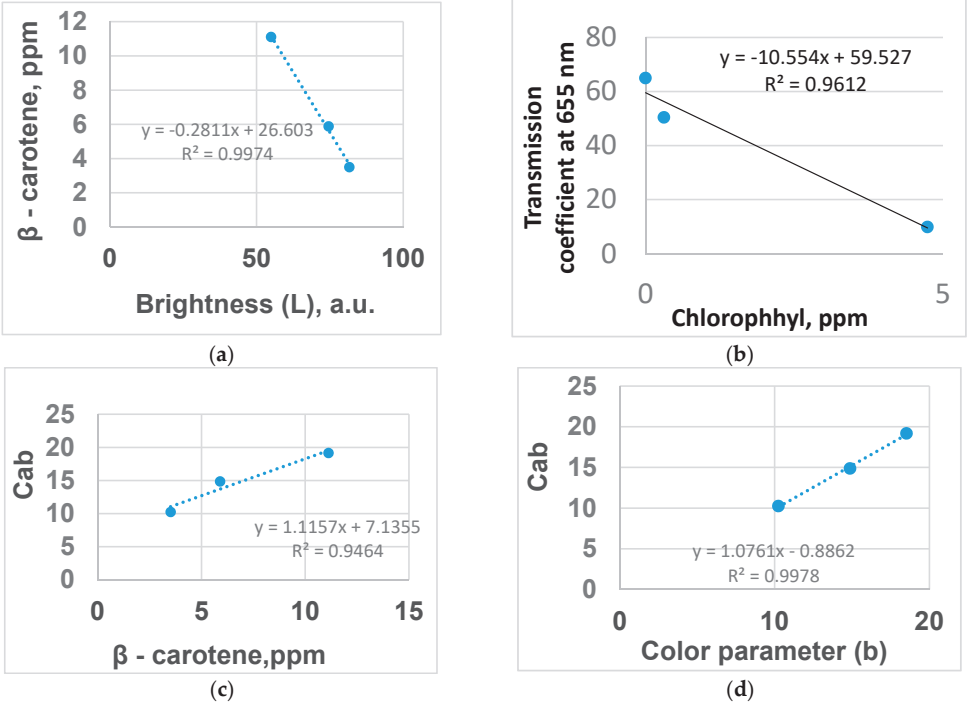
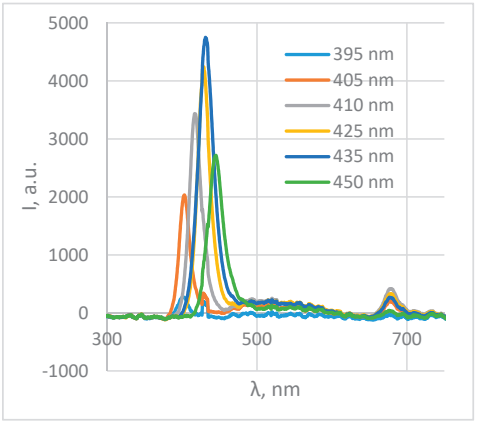


Figure 2. Linear dependances between contents of pigments and color characteristics. (**a**) β-carotene related to brightness; (**b**) Transmission coefficient at 655 nm related to chlorophyll; Cab related to: (**c**) β-carotene and (**d**) color parameter.

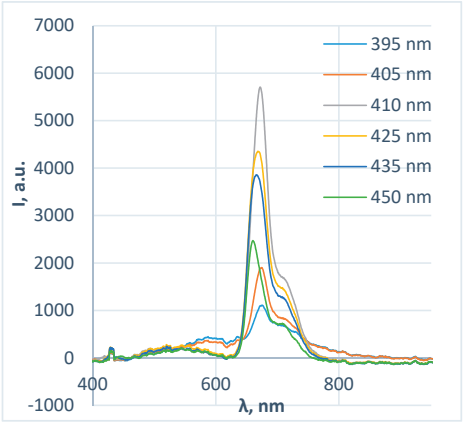
The fluorescence spectra of ethanolic extracts from *Sempervivum tectorum* L. with excitation light of 395 nm, 405 nm, 410 nm, 425 nm, 435 nm, and 450 nm were obtained. The results are presented in Figure 3. In addition, for better fluorescence visualization, excitation-emission matrices of ethanol extracts are shown in Figure 4.

For better visualization, in addition to the individual fluorescence spectra shown in Figure 3a–c, the three-dimensional emission matrices and two-dimensional contours with isolines are presented, representing a universal profile of the so-called fingerprints for selected samples extracts. The matrices presented were obtained after averaging the fluorescence spectra of the samples from a given concentration.

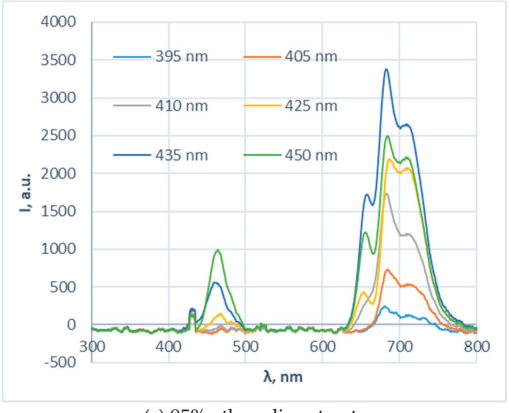
The contents of essential and toxic elements in three extracts were determined by ICP-MS. Results are presented in Table 4.



(**a**) 10% ethanolic extracts

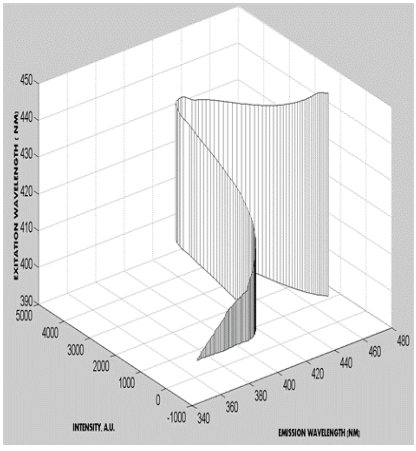


(**b**) 50% ethanolic extracts

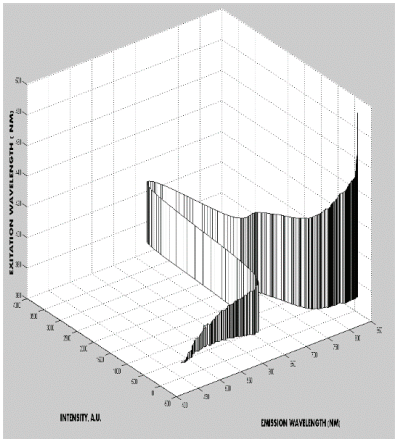


(**c**) 95% ethanolic extracts

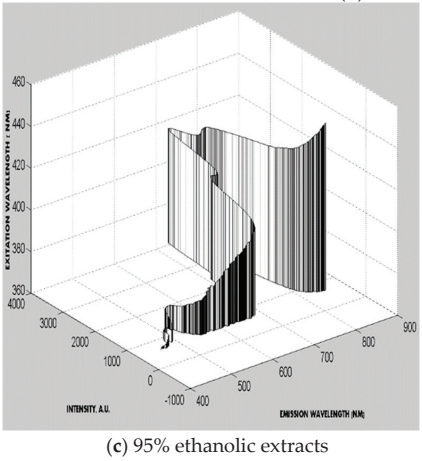
Figure 3. Fluorescence spectra in the visible region for water ethanol extracts from *Sempervivum tectorum* L.: (**a**) 10%; (**b**) 50%; (**c**) 95%.



(**a**) 10% ethanolic extracts



(**b**) 50% ethanolic extracts



(**c**) 95% ethanolic extracts

Figure 4. Excitation-emission matrices of ethanolic extracts of *Sempervivum tectorum* L. (**a**) 10%; (**b**) 50%; (**c**) 95%.

Table 4. Concentrations of essential and toxic elements in water-ethanolic extracts (RSD for all samples varied between 2–7%).

Ethanol, %	K, g kg^{-1}	Ca, g kg^{-1}	Mg, g kg^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}	Zn mg kg^{-1}	Cu mg kg^{-1}	Pb, mg kg^{-1}
10%	10.8	20.0	1.06	123	7.18	9.09	0.83	0.13
50%	11.9	2.96	1.01	35.2	1.46	2.24	0.93	0.07
95%	2.63	0.09	0.07	1.63	0.24	1.35	1.54	0.09
Ethanol, %	**Na, mg kg^{-1}**	**Al, mg kg^{-1}**	**Ba, mg kg^{-1}**	**Cr, mg kg^{-1}**	**Co, mg kg^{-1}**	**Ni, mg kg^{-1}**		
10%	13.2	4.53	16.4	0.16	0.19	0.68		
50%	26.0	2.57	0.84	0.09	0.05	0.36		
95%	10.9	1.26	0.05	<0.05	<0.02	0.04		

Tl, As, Cd, and Hg concentrations are below detection limit (0.02 mg kg^{-1}) for all extracts.

4. Discussion

The results from the total phenolic contents and antioxidant activities of the obtained plant extracts are summarized in Table 1. The highest total phenolic content was found in the 50% ethanolic extract about—0.84 ± 0.08 mg GAE/mL. This extract also possessed the highest antioxidant potential as evaluated by the DPPH and FRAP assays at about—7.02 ± 0.71 mM TE/mL and 6.15 ± 0.25 mM TE/mL, respectively. The antioxidant activity and total phenolic content in the 10% ethanol extract showed approximately two times lower values than in the 50% ethanol extract. The lowest antioxidant activity was found in the 95% ethanol extract. Therefore, the extraction of bioactive compounds depended strongly on the water content in a solvent. A similar observation was reported by Taneva et al. for water and hydroethanolic extracts from rosehip (*Rosa canina* L.) fruits [24]. In agreement with their results, our study demonstrated that the 50% ethanol extract had the highest antioxidant potential. The better extraction of phenolic compounds from dried and lyophilized leaves of *Sempervivum tectorum* with a water to ethanol ratio of 1:1 was also reported. The total phenols reached 15-40 mg GA/g dry weight [25]. As might be expected, a strong correlation was found between the total phenolic content and the antioxidant activity. A correlation coefficient above 0.9 was calculated for both the total polyphenols and antioxidant activities of extracts: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging ability and total polyphenols and FRAP (ferric reducing antioxidant power). Evidently, the antioxidant activity of the 50% ethanol extract was mainly due to the high level of total phenolic content in this extract. 50% ethanol extracts of *Sempervivum tectorum* could be successfully used for food and pharmaceutical purposes due to the safety of the solvent and as it is rich in polyphenols. As confirmation, Šentjurc et al. [26] explained that compounds in pure extracts of *Sempervivum tectorum* L. that possessed the highest antioxidant potential are oligomeric polyphenols. Knez Marevci et al. [25] showed the antioxidative potential of dried and lyophilized *S. tectorum* extracts only by the DPPH assay. Similar results for ABTS and DPPH radical scavenging assays used to evaluate the antioxidant potential of different extracts of *Sempervivum tectorum* leaves were observed by Alberti et al. [27]. In this study we demonstrated that the 50% ethanol extract showed better radical scavenging activity by the DPPH method than by the metal reducing activity (FRAP assay) (Table 1) *Sempervivum tectorum* 70 % (*v/v*) ethanol extract showed IC_{50} in concentration 74.5 ± 3.6 μM/mL (DPPH) [27], while in our case 50% ethanol extracts demonstrated much higher IC_{50} 3.45 mM TE/mL (DPPH). The correlation of total polyphenol content and radical scavenging activity (DPPH), as well as total polyphenol content and metal reducing activity (FRAP) was considered as highly significant with $r^2 = 0.9988$ and $r^2 = 0.9994$, respectively.

The color coordinates were obtained in two different colorimetric systems (Table 2). Color parameters x and y, which are connected with the purity of the color of samples from *Sempervivum tectorum* L., have been increased with higher ethanol contents. The uniform color space CIELab has been used to better present the color characteristics of the investigated extracts. The increase in the color parameter b proves that the yellow component dominates in 10% and 50%, intensifying with increasing ethanol content. In 95% ethanol extract the blue component dominates—the parameter b < 0—in contrast to 10% and 50% extracts.

The results presented in Table 3 show that as the ethanol content increases, so does the amount of β-carotene. Chlorophyll exists in 50% and 95% ethanol extracts, but it is absent in 10% extract (Figure 1). The absorption band between 630 nm and 690 nm is absent in the 10% ethanol extract of *Sempervivum tectorum* L. because it does not contain chlorophyll. It has the greatest transmission in the visible range at about 60%–70%. For 95% of the ethanol extracts from *Sempervivum tectorum* L., the transmission spectrum in the visible region has two regions—the first is in the range of 440 nm–480 nm, and the second is between 610 nm–660 nm. There is a linear dependence between the transmission coefficient at 655 nm and the content of chlorophyll.

The main compounds associated with the antioxidant effect of *Sempervivum tectorum* L. extracts may also correlate with the fluorescence, absorption, or transmission spectra of the samples.

The fluorescence spectra of 50% and 95% ethanolic extracts of *Sempervivum tectorum* L. were similar (Figures 3 and 4). An intense fluorescent maximum in the range of 675–690 nm was observed, which was associated with the presence of chlorophyll. It is the most intense and clearly expressed for the 50% ethanol extract. The main compounds associated with the antioxidant activities were carotenoids and polyphenolic compounds (especially phenolic acids and flavonoids). In our case, the fluorescence spectra provide a fluorescence maximum in the range of 630–700 nm, which is associated with the presence of chlorophyll in the samples. For the sample with the 50% ethanol content, there is a low-intensity fluorescent band in the range of 500–580 nm, associated in the literature with the presence of β-carotene.

Analysis of the transmission spectra shows that intense absorption bands between 630 nm and 690 nm were observed only in 50% and 95% water-ethanolic extracts and were absent in the 10% ethanolic extract of *Sempervivum tectorum* L. (chlorophyll is not leached in this extract).

Several correlations have been established between the parameters of applied photonics and the contents of pigments and with antioxidant activities and total phenolic contents.

$$\text{DPPH} = 8.405 * \text{TPC}, R^2 = 0.991 \quad (1)$$

$$\text{FRAP} = 7.4975 * \text{TPC}, R^2 = 0.991 \quad (2)$$

$$\beta\text{-carotene} = 0.9026 * \text{b} - 6.2895, R^2 = 0.923 \quad (3)$$

$$\text{T} = -0.554 * \text{Chlorophyll} + 59.527, R^2 = 0.961 \quad (4)$$

Essential element content in studied extracts varied considerably. The concentrations of elements like K and Na showed relatively high correlation coefficients above 0.8 with total phenolic content showing most like the chemical compositions of phenols. Unexpectedly, content of elements like Fe, Cu, and Zn do not correlate with total phenols although it is well known that Fe, Zn, and Cu formed stable complexes with phenols. Most likely parts of these elements are bound to other constituents of investigated extracts. As can be seen from Table 4, the ethanol concentration significantly affects the extraction of elements such as Ca, Fe, Mn, Zn, Al, Co, Ba, and Cr. Their contents in the extracts decrease with the increase in ethanol content. Only the concentration of Cu increases with the increase in ethanol content.

Toxic element levels in prepared extracts/infusions are under regulation at the national or regional level. Permissible limits for toxic elements in extracts have been compared by several authors [28]. According to the World Health Organization, the permissible limit for cadmium concentrations in herbal products is 0.3 mg kg^{-1} Cd and for lead it is 10.0 mg kg^{-1} Pb [29]. Results obtained for all studied extracts showed very low concentrations for toxic elements, much below the permissible limits. Furthermore, the results for highly toxic elements like Cd, Tl, As, and Hg are below the detection limit of 0.02 mg kg^{-1} for all studied extracts. As a general rule, the 95% ethanol extract has the lowest concentrations of elements, suggesting that chemical species of essential and toxic elements in *Sempervivum tectorum* L. are aqueous soluble and highly bioavailable.

5. Conclusions

The highest total phenolic content 0.84 ± 0.08 mg GAE/mL was found in 50% ethanol extract. This extract also has the highest antioxidant potential as assessed by DPPH and FRAP assays. High correlation coefficients were found between total phenolic contents and antioxidant activities of water-ethanolic extracts from *Sempervivum tectorum* L. The same is valid for the strong relationship between phenolic contents and concentrations of Na and K. Most likely bioavailable species of elements such as Fe, Zn, Ca, and Mg are mostly aqueous

soluble. On the contrary, concentrations of compounds like β-carotene and chlorophyll increase with the increase in ethanol in the extract. The contents of toxic elements, As, Cd, Tl, and Pb, are far below the permissible limits for pharmaceutical products. Results from this study may be used for preliminary prognosis of pharmaceutical applications of extracts from *Sempervivum tectorum* L.

Author Contributions: G.G. and I.K. constructed and conceived the project. K.N. and N.P. designed the study. G.G., I.K., K.N., S.M. and N.P. performed the study. V.A. and I.M.-T. analyzed the data and interpreted the results. K.N., G.G. and N.P. wrote the article. All authors have read and agreed to the published version of the manuscript.

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Chemical Composition and Anti-Microbial Activity of Hog Plum (*Spondias mombin* L.) Peel Oil Extracted from Different Regions of Tropical Climates

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Abstract: The hydro-distilled essential oil of hog plum peel may be used for enhancing the flavor and taste of food products as well as for hiding the unpleasant odor of drugs. Thus, the waste peels of *Spondias mombin* appear to have economic importance. To find out the chemical composition and anti-microbial properties of hog plum peel oil, the samples were collected from different regions of Bangladesh for extraction and identification of volatile compounds by GC-MS, where dichloromethane was used as an extraction solvent. The required standard analytical methods were used to assay the anti-microbial properties of hog plums. In this study, pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-oxiranen (29.04%), (3,3.1,1)-4-dimethylethyl-1,1-phenol (8.00%), cycohexanol-3 (10.85%), 4-hydroxy-penzeneethanamine (7.09%), hydroxylamine (4.63%), dibutyl phthalate (6.85%), etc., were majorly determined. Consequently, the highest content of 75.81% volatile compounds was found in the Dinajpur district, where the lowest content of 35.00% was found in the Rajshahi district. In contrast, 33 volatile compounds were identified in hog plum peels collected from the Barishal district, whereas 22 compounds were detected in the peel samples collected from the Dinajpur district. In addition, the antimicrobial activity of the oil was analyzed by the disk diffusion method, and the results revealed that the highest Ciprocin content was recorded in the hog plums of Barishal (22.0–23.0 mm), while the lowest was recorded in the Mymensingh sample (20.67–21.63 mm), which was on par with Rajshahi sample (20.70–21.50 mm). The results of the anti-fungal activities of the peel oil showed the highest zone of inhibition against the *Aspergillus niger* (11.63 $\pm$ 0.0003 mm) and *Penicillium oxalicum* (13.67 $\pm$ 1.97 mm) content of the Rajshahi and Pabna district samples, respectively.

Keywords: waste peel; essential oil; GC-MS; volatile compounds; antimicrobial activity; chemical composition

1. Introduction

Hog plum (*Spondias mombin* L.) is a member of the Anacardiaceae family and is locally known as "Amra" in Bangladesh. Its fruit is a drupe characterized with a mixed taste of sour and sweet, and has gained increased importance in modern medicine for its possible pharmacological activities [1,2]. It grows mostly in the Indian subcontinent, e.g., in Bangladesh, India (Assam and Bombay), and Nepal. In Bangladesh, the cultivatable area of hog plum was about 1247 acres, and the corresponding total production was 36,068 metric tons (MT) in 2016–2017 [3,4]. Hog plum is mainly consumed as fresh fruit and has other uses such as making jam, jelly, squash, and marmalades, on a small scale and on a commercial scale. After the consumption and manufacturing of the above products, the peels are discarded as waste that holds almost 20% of the fresh fruit [5,6]. These wastages are considered a problem for the food processing industry and pollution monitoring organizations [7]. Nevertheless, these waste portions could be used as a potential source of valuable by-products like essential oils (EOs) which could be extracted from flowers, leaves, stems, roots, seeds, barks, resins, or fruit rinds [6].

Essential oils from citrus fruits are a group of natural flavors and fragrances which are popularly used in the food and pharmaceutical industries, daily chemical products, and in the health care field [8–12]. The products of medicinal plants like EOs and their antimicrobial properties have been empirically recognized for centuries, but recently the antimicrobial properties have been studied and confirmed scientifically [13–15]. These EOs effectively control the growth of different microorganisms like fungus, yeasts, bacteria, etc., which has been reported in several studies [16–21]. The bark extract of hog plums exhibits a valuable antibacterial activity, while the aqueous extract of the bark has shown a moderate antibacterial activity against *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio cholerae* [22,23].

Many studies have been carried out regarding the fruit, bark, and leaf extracts of hog plums [24–28], but there has been little research work on hog plum peels, which may contain various bioactive volatile and antimicrobial compounds. The extraction and identification of bioactive volatile compounds (VCs) in hog plum peels and their preservation are a potential source of medicine as well as nutrients for functional foods and feed industries.

Therefore, the present study was conducted to discover the chemical composition and anti-microbial properties of hog plum peel oil collected from different regions of Bangladesh.

2. Materials and Methods

2.1. Sample Collection

The samples of *Spondias mombin* were collected in the early summer season from the local market of the Dinajpur (Latitude: 25.6221° N, Longitude: 88.6438° E, and Altitude: 42.0 m), Mymensingh (Latitude: 24.7471° N, Longitude: 90.4203° E, and Altitude: 19.0 m), Barishal (Latitude: 22.7010° N, Longitude: 90.3535° E, and Altitude: 1.22 m), Rajshahi (Latitude: 24.3745° N, Longitude: 88.6042° E, and Altitude: 18.0 m), and Pabna districts (Latitude: 24.0023° N, Longitude: 89.1413° E, and Altitude: 19.0 m) of Bangladesh. Three separate samples were collected from each district, which were treated as three replications. The microorganism species used in this study were bacteria (ATCC) such as *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and fungi such as *Aspergillus niger* and *Penicillium oxalicum*. The pure cultures of these species were obtained from the Laboratory of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh. The cultures of each microorganism were inoculated on nutrient agar (NA) and Sabouraud dextrose agar (SDA) (Merck KGaA, 64271 Darmstadt, Germany) at 37 °C for 24 h, and stored at less than 4 °C.

2.2. Preparation of Hog Plum Peel Powder

The samples were washed and rinsed with deionized water and subsequently peeled with a knife carefully. The peels of each of the five samples were weighted and spread in five different trays for drying in a cabinet dryer (Model FMA-275) at 60 °C for three days until they were completely dehydrated. Then the samples were taken out from the drier and put into desiccators for a few minutes to adjust to the ambient temperature. Then, the dried samples were blended by an electric blade blender (Vitamix 5200 Series) to make the powdery form and to prepare the peel samples for hydro-distillation.

2.3. Extraction of Essential Oil

Five samples of hog plum peels were collected in mid-June 2018 from the five districts. Each sample of peel powder, weighing 20 g, was suspended in 300 mL of deionized water and subjected to steam distillation using a Clevenger-type apparatus for 4 h. Then the sample oils were collected, dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and stored in sealed vials under refrigeration at 4°C until analysis [29]. The yield (%) of oil was calculated by the following formula:

$$\text{Yield (\%)} = (\text{Weight of oil})/(\text{Weight of the fresh sample}) \times 100$$

2.4. Gas Chromatography and Mass Spectrometry (GC-MS) Analysis

GC-MS was conducted with a Varian Saturn 2200 equipped with an ion trap detector (ITD) for the identification of different components of essential oil. To obtain better results, dichloromethane was used as solvent. The sample of 2.0 µL was injected on a DB-5 MS (30 m, 0.25 mm ID, 0.25 µm film thickness) column. Helium was used as a carrier gas with a flow rate of 1 mL/min and a split ratio of 1:5. The temperature in the oven-dryer was set at 50 °C for 1 min, followed by a temperature gradient of 2.5 °C/min to 280 °C/min for 40 min. The injector and transfer line temperatures were set to 250 °C and 280 °C, respectively. Various components were identified by their retention time (5.52–22.34 min) and peak enhancement with standard samples in gas chromatographic mode and a National Institute of Standards and Technology (NIST 20) library search from the derived mass fragmentation pattern of various components of the essential oil [30].

2.5. Determination of Antimicrobial Activity

The antimicrobial activity of the tested essential oil was monitored using the disc diffusion method [31] against different food-borne pathogens including bacteria (*Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*), and two selected fungi (*A. niger* and *P. oxalicum*). The antibacterial and antifungal screening was performed briefly using ciprofloxacin (10 µg/disc) and fluconazole (10 µg/disc) as a positive control and sterile water as a negative control. Standard culture media of each type of bacteria and fungi were employed on NA and SDA plates (100 mL each), where 5 µL (1000 ppm) of the essential oil was used for each test sample. During the investigation, the incubation temperature was maintained for both fungi (25 °C) and bacteria (37 °C). The zones of inhibition thus developed against the tested microorganisms were measured after a period of 48 and 96 h. All experiments were conducted in triplicate. The results of the antimicrobial activity of the peel oil against the different microorganisms were expressed as resistant, intermediate, and sensitive.

2.6. Statistical Analysis

Antibacterial and antifungal experiments were performed in triplicate and the analyzed data were presented as mean ± SE. The obtained data were subjected to one-way ANOVA using MS Office 2007 (significance: $p \leq 0.05$; coefficient interval (CV): >95%).

3. Results and Discussion

3.1. Yield

The yield of essential oil by the hydro-distillation method was satisfactory from the *Spondias mombin* peel samples collected from the five selected districts of Bangladesh (Table 1). However, the Barishal sample showed the highest production of EOs (75%), while the Pabna sample showed the lowest value (47%). Based on the yield of essential oil, the samples can be ranked as Barishal > Dinajpur > Mymensingh > Rajshahi > Pabna. Natural plant-derived non-phytotoxic substances such as EOs may increase the shelf-life of processed food products by destroying the cell wall of bacteria and fungi. Thus, researchers have devoted their interests to producing natural medicinal and value-added food products from plant-based extracts. The experimental volatile oils of hog plum peels exhibited strong flavor alike to that of the fresh raw samples. Table 1 shows a higher yield of oil from the peels than that obtained from the fruits, leaves, and barks of hog plums [32]. On the other hand, *Mangifera indica* is taxonomically close to *S. mombin* which also had similar results [33,34]. The yield was comparable to that reported in previous studies. Oven-dried citrus peels exhibited higher oil yield followed by the ambient-dried and fresh samples. Soumaya et al. [35] also reported that the yield of volatile compounds varied during ripening and reached the maximum values during the middle stage of maturity (second stage) for citrus fruits, while the highest lemon yield was determined at the beginning of fruit maturation and decreased thereafter.

Table 1. Regional samples, oil mass, and its product percentages.

Regional Sample	Peel Mass (g)	Oil Mass (g)	Yield (%)
Barishal	20	0.15	75
Dinajpur	20	0.14	70
Mymensingh	20	0.136	68
Rajshahi	20	0.118	59
Pabna	20	0.094	47

3.2. Chemical Composition of Volatile Oil

There were 95 chemical compounds in the collected samples of hog plum peels (Table 2), and among them, the esters, alcohols, ketones, aldehydes, acids, hydrocarbons, phenols, and others comprised 26 (27.37%), 23 (24.21%), 16 (16.84%), 9 (9.47%), 6 (6.32%), 4 (4.21%), 5 (5.26%), and 6 (6.32%) of these compounds, respectively. Among the total (95) volatile compounds (VCs) of the five light yellowish oil samples, the Barishal sample oil was found to be rich in pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-oxiranen (29.04%), and 5-heptone-methyl-one, 6-2 ester (3.24%); the Dinajpur sample oil contained pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-xiranen (21.57%), cycohexanol-3 (8.59), picolylamine (10.49%), (3,3.1,1)-4-dimethylethyl-1,1-phenol (8.00%), 4-hydroxy-penzeneethanamine (7.09%), and dibutyl phthalate (4.32%); the Mymensingh sample oil contained pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-oxiranen (28.52%), cycohexanol-3 (10.85%), dibutyl phthalate (6.85%), and cyclotetrasiloxane (1.94%); the Rajshahi sample oil contained pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-oxiranen (9.27%), cycohexanol-3 (5.80%), borneol (2.35%), and hydroxylamine(4.63%); and the Pabna sample oil was found to be rich in pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-oxiranen (11.45%), cycohexanol-3 (25.00%), borneol (4.96%), dibutyl phthalate (3.03%), and 3-cyclohexen-1-ol (3.57%). Furthermore, pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-oxiranen was detected in all the samples, but cycohexanol-3 was present only in the Mymensingh, Dinajpur, Rajshahi, and Pabna samples, and was absent in the Barishal samples. (Methylyethyl-1)-4-methyl-oxabicyclo, 1-7 was present in the Dinajpur, Barishal, and Pabna samples but was absent in the Rajshahi and Mymensingh samples. Cycohexanol-3 was present in the Mymensingh, Rajshahi, Dinajpur, and Pabna samples but was absent in the Barishal sample. Dibutyl phthalate was present in the Dinajpur,

Pabna, and Mymensingh samples, but was absent in the Barishal and Rajshahi samples, and so on.

Table 2. Chemical compounds obtained from hog plum samples by GC-MS (Retention time: 5.52–22.34 min).

Sl. No.	Chemical Compound	Amounts (%)				
		Barishal	Dinajpur	Mymensingh	Rajshahi	Pabna
1	Propen 1, 2, 3 trichloro benzene	0.24	-	-	-	-
2	Epoxycarane-3, 2	0.85	-	-	-	-
3	Chloride-butyl-benzene, tert	0.47	-	-	-	-
4	Pentenyl-3-thy-met-4-alpha-methyl-alpha-ethanol-oxiranen	29.04	21.57	28.52	9.27	11.45
5	Acetate, -benzenethanol, alpha, dimethyl	0.14	-	-	-	-
6	Methyl-nonyne, 7-1	0.12	-	-	-	-
7	(Fenchol)-trimethyl-ol, 1, 3, 3-2-bichlo(2.2.1)hepton	0.17	-	-	-	-
8	(Methylethyl-1)-4-methyl-oxabicyclo(4.1.6) hepton, 1-7-	1.37	-	-	-	-
9	Nonennal, (E)-6	0.64	-	-	-	-
10	(Methylyethyl-1)-4-methyl-oxabicyclo, 1-7	1.37	0.56	-	-	2.13
11	3,1-Cycloheptadiene	0.44	-	-	-	-
12	(Yl-buten 3-1)-bicyclo (2.2.1) hepton, 2	1.44	-	-	-	-
13	10-Methyl-8-ol acetate-1-tetradecen	0.49	-	-	-	-
14	4,1-Cyclohexadiene-(methyl-1)-methanol,4-1	0.49	-	-	-	0.39
15	Exo-2-hydroxycineole	0.36	-	-	-	-
16	5-Heptone-methyl-one, 6-2 ester	3.24	-	-	-	-
17	(Ethyl-methyl-1)-benzaldehyde,4	0.28	-	-	0.04	0.01
18	Methyl-bromo-ene,7-7-pentadec	0.26	-	-	-	-
19	(Ethyl-methyl-1)-1-methyl-ene,4-2-bicyclo(3.1.0)hexan-	0.92	-	-	-	-
20	(Ethyl-methyl-1)-5-methyl-phenol,2	0.73	0.29	0.14	0.04	0.31
21	Terpenyl acetate-alpha-epoxy	0.83	-	0.12	1.36	0.61
22	Hylidenne-methanol,2-6, bicyclo (3.1.0) hexane	0.53	-	-	-	-
23	9-E-8-Methyl-ol, acetate-2-tridecel	0.71	-	-	-	-
24	15,12,9-bis (a)-octadecatrienoicacid, 2,3	0.09	-	-	-	-
25	Diene, 2-[8, (7) 1]-menpha-p-2r,4r-hydroperoxide	0.58	-	-	-	-
26	8-Hydroxycarvctancetone	0.08	-	-	-	-
27	2,5-Dihydro-3,4-furanacetic acid	0.38	-	-	-	-
28	3-Buten-2-one-4[2,6,6-trimethyl-1-cyclohexen]	0.11	-	-	-	-
29	2-(3,4-Dibromo-4-methyl cyclohexyl) propanol	0.20	-	-	-	-
30	Phenol, 2,4-bis (1.1 dimethylehyl)	0.06	0.68	0.46	-	0.30
31	Nonadecane	0.11	-	-	0.65	-
32	Hydroxylamine-O-decylamine	0.09	-	-	-	-
33	Phthalic acid, isobutylnonyl ester	0.96	-	0.45	0.46	-
34	Cycohexanol-3	-	8.59	10.85	5.80	25.00
35	Cycohexanol, 5-methyl-2(1-methylethen)	-	0.80	-	-	-

Table 2. *Cont.*

Sl. No.	Chemical Compound	Amounts (%)				
		Barishal	Dinajpur	Mymensingh	Rajshahi	Pabna
36	Isopulegol acetate	-	0.50	-	-	1.91
37	Borneol	-	0.82	-	2.35	4.96
38	3-Acetoxy-p-menthane-3-one	-	0.41	-	-	-
39	2-Methyl-3-(1-methylethyl cyclohexanol)	-	0.18	-	0.89	-
40	Carbamic acid, N-(1,1-ethyl bistrifluoron)	-	0.82	-	-	-
41	(3,3.1, 1)-4-Dimethylethyl-1,1-phenol	-	8.00	-	-	-
42	Picolyamine	-	10.49	-	-	-
43	3,4-Methyl-dimethyl 2,3-butyryl benzoate	-	0.57	-	-	-
44	Phthalicacid, 2-acetylphenyl heptyl ester	-	0.44	-	-	-
45	2-Phenylquinazolin-4-ol	-	1.38	-	-	-
46	Dibutyl phthalate	-	4.32	6.85	-	3.03
47	4-Hydroxy-penzeneethanamine	-	7.09	-	-	-
48	2,4-Dimethyl-5,6,11,12 tetraaza	-	2.71	-	-	-
49	4-Oxo-1,2,3,4,7,12-octahydropy-	-	1.46	-	-	-
50	2,2-(Dimethyl-1,1-bisene-6-methylethyl-phenol	-	3.84	-	-	-
51	4-(1-methylethyl) benzenmethanol	-	0.29	0.29	-	-
52	Cyclotetrasiloxane	-	-	1.94	-	-
53	Benzene, 1-methyl-4(1-methylethenyl)	-	-	0.07	-	-
54	1,7-Octaden-3-ol, 2,6-dimethylamin	-	-	0.07	-	-
55	Decamethylcyclopentasiloxane	-	-	0.31	-	-
56	1,3,3-Trimethylbicyclo[2.2.1]-heptan-2-ol propanoate	-	-	0.47	-	-
57	3-Cyclohexen-1-ol	-	-	0.32	0.73	3.57
58	Isopuleggol acetate	-	-	1.87	0.24	1.22
59	Borneal	-	-	0.78	-	-
60	Bicyclo (3.1.0) hexan 4-methyl-1	-	-	1.30	-	-
61	P-menth-2-en-7-ol, trans	-	-	0.81	-	-
62	Nonynoic acid, 7-methyl ester	-	-	0.34	-	-
63	5,7-Dodecadiyl, 1,12-diol	-	-	0.32	-	-
64	O-decyl-hyroxylamine	-	-	0.21	-	-
65	Hyroxylamine, O-decyl-	-	-	0.28	-	-
66	Acetic acid, Chloro-albydrate	-	-	0.24	-	-
67	(E)-2-Octenal	-	-	-	0.03	-
68	7-methyl-1-Nonyne	-	-	-	3.52	-
69	Exo-Fenchol	-	-	-	0.11	-
70	2,4-Pentadien-1-ol,3-pentyl-, 2Z	-	-	-	0.73	-
71	Isobornyl formate	-	-	-	0.15	-
72	Sanitolina alcohol	-	-	-	0.78	-
73	1,3,3,-Trimethyl-2-oxabicyclo[2,2,2] octan-6-ol	-	-	-	0.18	-
74	2-Octen-1-ol, 3,7 –dimethyl –isobutyrate	-	-	-	0.12	0.06
75	(E)-8-Methyaltetradec 1-ol acetate	-	-	-	0.37	-

Table 2. *Cont.*

Sl. No.	Chemical Compound	Amounts (%)				
		Barishal	Dinajpur	Mymensingh	Rajshahi	Pabna
76	10 Methyl-8-tetradecen-1-ol acetate	-	-	-	1.69	-
77	Phthalic acid, 4-butyl-octyl ester	-	-	-	0.69	-
78	Hydroxylamine	-	-	-	4.63	-
79	2-Methyl-2-propenyl benzene	-	-	-	0.17	-
80	1-Pentadecyne	-	-	-	-	0.56
81	Bicyclo (1,2,2) heptan-2-ol, 1,3,3 trimewthyl-	-	-	-	-	0.53
82	(E)—6 Nonenal	-	-	-	-	0.77
83	Beta-cisterpineol	-	-	-	-	0.56
84	Trifluoro-epiiso-bomeol	-	-	-	-	0.21
85	Santolina alcohol	-	-	-	-	1.46
86	1,5,5-Trimethyl—6-methylene cyclohexen	-	-	-	-	0.30
87	E-3 Bicyclo[2.1.1] trihepten	-	-	-	-	0.91
88	Trans-m-2,8-mentha –dienol	-	-	-	-	0.22
89	Bicyclo [3.1.0] hexane 2-one 5,5,6-trimethyl-	-	-	-	-	0.96
90	Bicyclo [3.1.0] hexane, 6-(1-isopropylidene)	-	-	-	-	1.19
91	1-Methyl-3-(1-methylethenyl) cyclohexane	-	-	-	-	0.67
92	Eugenol	-	-	-	-	0.92
93	2,4,4-Trimethyl-1,5-dienyl cyclohexane	-	-	-	-	0.16
94	4-Hexyl 2,5-dihydro-3-acetic acid	-	-	-	-	0.15
95	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	-	-	-	-	0.78
	Not identified (N.I)	51.72	23.90	42.23	64.38	34.42
	Identified	47.79	75.81	57.01	35.00	65.30
	Total (%)	**99.51**	**99.23**	**99.24**	**99.38**	**99.72**

Significant spatial variations were observed in the number of VCs detected in the hog plum peel samples (Figures 1–3). The number of VCs varied among hog plum peel samples (Figure 1 and Table 2). The highest number of VCs (75.81%) was recorded in samples collected from Dinajpur followed by those collected from Pabna (65.30%), Mymensingh (57.01%), and Barishal (47.79%), while the lowest number of VCs (35.00%) was observed in the samples collected from Rajshahi. Figure 2 represents the number of VCs, which were identified by GC-MS analysis of *S. mombin* from the five districts of Bangladesh. The numerical values of the chemical compounds of hog plum peel essential oil from Dinajpur, Mymensingh, Barishal, Rajshahi, and Pabna were 22, 23, 33, 24, and 30, respectively. It was reported earlier that the number of VCs present in the essential oils of hog plum peels is comparatively higher than that of mango peels [34]. This is in agreement with the earlier literature studies, which showed a considerable variation in the chemical composition of peel EOs concerning varieties and drying conditions [36]. The investigational components of the oil were higher in number than those of earlier literature which was held on the fresh fruits, leaves, and barks of hog plum cultivated in the tropical conditions of Brazil [37]. Several studies also considered the volatile oil of mango fruits, leaves, and peels with results comparable to those of hog plum peel oil. Each of the volatile oils exhibited different chemical constituents that may be attributed to several factors such as ecological and climatic conditions, plant age, and genotypic characteristics [38–43]. The

esters showed the highest value (27.37%), and hydrocarbons showed the lowest value (4.21%) among the volatile substances in this study. Phthalic acid, isobutyl nonyl ester, and 2-phenylheptyl ester-acetyl-phthalic acid were detected in lower quantities. These substances, produced biosynthetically from unsaturated fatty acids, are precursors for the straight-chain esters [44]. Dichloromethane was used because generally, it appears as the best solvent for extraction of a wide class of flavors. The GC-MS analysis revealed that the hog plum peel oil contained different kinds of chemical compounds and/or EOs (Table 2) which have numerous antimicrobial properties that efficiently regulate the growth of different microorganisms such as fungus, bacteria, and yeasts, as envisaged earlier in several studies [21,22]. It has been proven that these compounds are potentially useful additives for food preservation against mycotoxigenic fungi and bacteria. A similar conclusion was suggested from a recent study, which ascertained that these additives prolong the shelf life and improve the quality of stored food products [45].

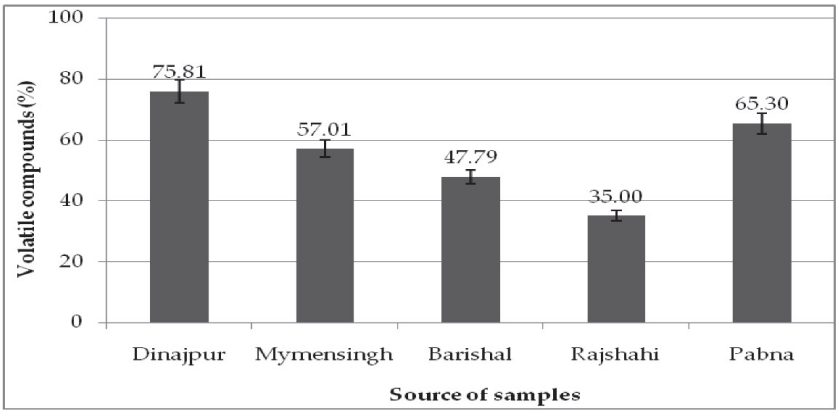

Figure 1. Yield of volatile compounds from hog plum peel samples collected from different districts of Bangladesh.

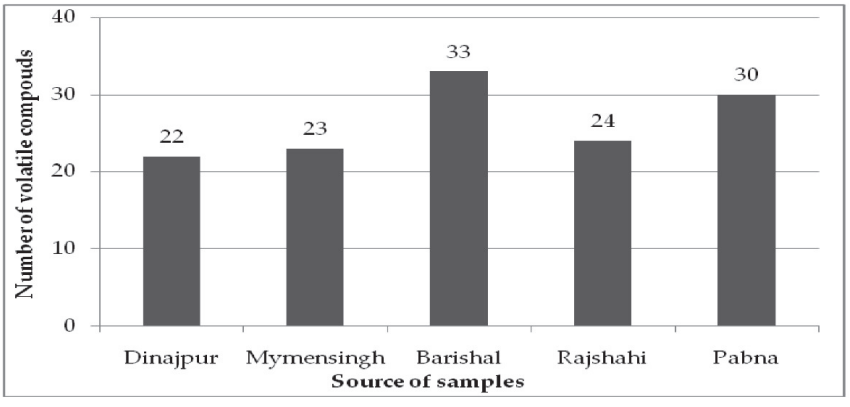

Figure 2. Number of volatile compounds identified from hog plum peel samples collected from different districts of Bangladesh.

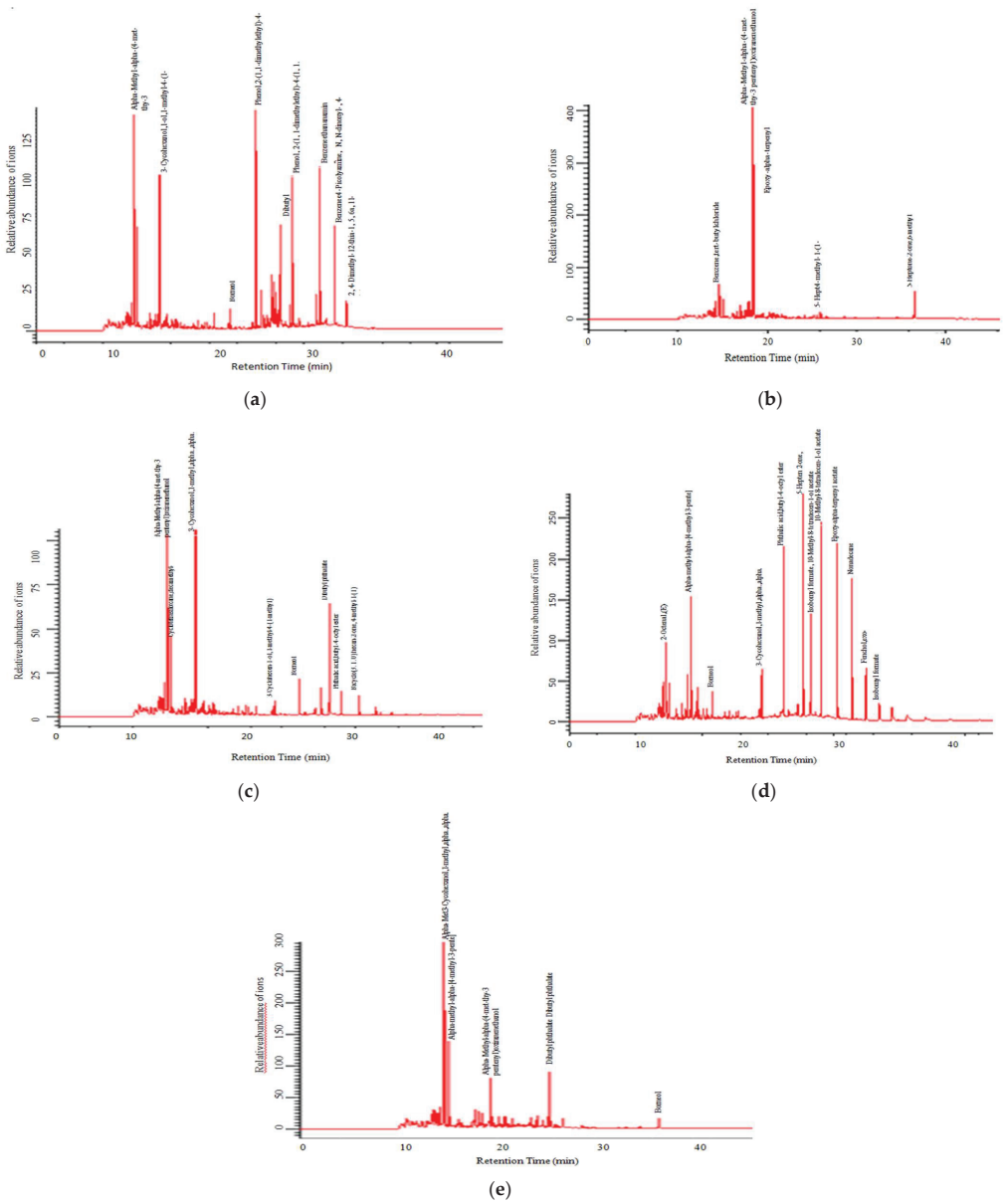

Figure 3. GC-MS chromatogram of *S. mombin* peel oil collected from (**a**) Dinajpur, (**b**) Barishal, (**c**) Mymensingh, (**d**) Rajshahi, and (**e**) Pabna districts.

3.3. Anti-Bacterial Activities of Essential Oil

In Table 3, antibacterial activity of peel oil was determined against three bacterial pathogens, namely, *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*. The disk diffusion method was used to determine the antimicrobial activity by measuring the zone of inhibition. All peel oils exhibited a moderate clearance zone of inhibition against *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*. Among those, the Barishal sample oil showed the highest antibacterial sensitivity. From the experimental data, the Barishal

sample oil showed inhibition diameters of 14 ± 0.17 mm, 13 ± 0.29 mm, and 12 ± 0.17 mm for *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* spp., respectively. However, the standard Ciprocin exhibited anti-bacterial activity ranging from 22 to 23 mm. The obtained oil samples showed significant or moderate antimicrobial and antifungal activity against clinically isolated pathogenic microbial strains in comparison to standard Ciprocin and fluconazole; hence, it might be considered essential to their potential for maintaining hygienic, healthy conditions. These results are similar to those of Chacko and Estherlydia [7]. Ciprocin (Ciprofloxacin) is a fluoroquinolone antibiotic used to prevent bacterial infections such as bone and joint infections, intra-abdominal infections, certain types of infectious diarrhea, respiratory tract infections, skin infections, typhoid fever, urinary tract infections, etc. Results from the third disk diffusion test of the peel oil, represented in Table 3, show that the gram-positive *S. aureus* bacteria was more sensitive to the tested extracts than the other two gram-negative bacteria. Most studies have investigated the action of EOs against food spoilage microorganisms and food-borne pathogens and showed that these oils are slightly more active against the gram-positive than the gram-negative bacteria [46]. They studied the resistance against bacteria and represented the inhibition zones against the ciprofloxacin and erythromycin. These results are comparable to those of our research. Kalemba et al. [47] classified the antimicrobial activity of EOs into three levels: weak activity (inhibition zone $\leq$ 12 mm), moderate activity (12 mm $\leq$ inhibition zone $\leq$ 20 mm), and strong activity (inhibition zone $\geq$ 20 mm), while our samples showed moderate to strong antimicrobial activity (Table 3). Moreover, several researchers have studied the antifungal activities of important *Spondias* species [48].

3.4. Anti-Fungal Activity of Essential Oil

As shown in Table 4, the antifungal activity of the peel oil samples was determined against two fungal pathogens, namely, *A. niger* and *P. oxalicum*. The disk diffusion method was used to determine the bioactivity by measuring the zone of inhibition. From our research data, peel oil from the Pabna sample produced satisfactory inhibition against *A. niger* and *P. oxalicum*. However, the highest inhibition zone of *P. oxalicum* (13.67 ± 1.97 mm) was observed against the Pabna sample oil, whereas *A. niger* showed the best inhibition against the Rajshahi sample oil (11.63 ± 0.0003 mm). The Barishal sample oil showed the second-highest zone of inhibition against *P. oxalicum* (13.17 ± 0.17 mm) followed by *A. niger* (11.33 ± 0.17 mm). On the other hand, standard fluconazole exhibits anti-bacterial activity ranges from 19 to 20 mm. The antifungal results of all the essential oil samples in our study were similar to the results of previous research [49]. The monoterpenes of the volatile oils were mainly terpene hydrocarbons that have antimicrobial activity [50–53]. These monoterpene hydrocarbons inhibit both bacteria and fungi via interference with spore germination and mycelia growth [54–56]. Most of the terpenoids and their derivatives found in this study are important natural medicinal chemical constituents with wide biological activities [33]. The phenolic compounds can donate a hydrogen atom to the free radicals, thus breaking the propagation of chain reactions during the lipid oxidation process [57,58]. The oil can also inhibit the activity of protective enzymes and sequentially inhibit one or more biochemical pathways [11]. Lu et al. [59] explained the effect of oxygen availability on the antimicrobial efficacy of the oil on *Staphylococcus aureus* and *Salmonella enteritidis*. Microaerobic or anaerobic conditions were greatly enhanced when these organisms were incubated. The antimicrobial components of the essential oil cross the cell membrane, interact with the enzymes and proteins of the membrane, and hence produce a flux of protons towards the cell exterior, which induces the changes in the cells and ultimately, promotes their death. Due to the presence of monoterpenes in EOs, they may be used extensively as natural preservatives in many food products, soaps, soft drinks, cosmetics, and perfumes for their lemon-like flavor and odor [48].

Table 3. Antibacterial activity of hog plum (*Spondias mombin* L.) peel oil extracted from different regions of Bangladesh.

SN	Bacterial Strains	Diameter of Zone of Inhibition (mm) *									
		BS		DS		MS		RS		PS	
		Cultures (5 µL/Petridish)	Standard (Ciprocin) (10 µg/disc)	Cultures (5 µL/Petridish)	Standard (Ciprocin) (10 µg/disc)	Cultures (5 µL/Petridish)	Standard (Ciprocin) (10 µg/disc)	Cultures (5 µL/Petridis)	Standard (Ciprocin) (10 µg/disc)	Cultures (5 µL/Petridish)	Standard (Ciprocin) (10 µg/disc)
1	*Salmonella* spp.	12.00 ± 0.17	23.00 ± 0.58	11.50 ± 0.01	22.5 ± 0.007	10.87 ± 0.015	21.63 ± 0.01	10.50 ± 0.007	21.50 ± 0.007	12.80 ± 0.03	22.00 ± 0.20
2	*Staphylococcus aureus*	14.00 ± 0.17	22.00 ± 0.27	13.83 ± 0.04	21.96 ± 0.0004	13.13 ± 0.001	21.00 ± 0.007	13.40 ± 0.190	21.40 ± 0.009	13.90 ± 0.0001	21.87 ± 0.05
3	*Escherichia coli*	13.00 ± 0.29	22.00 ± 0.22	12.7 ± 0.015	21.26 ± 0.019	12.37 ± 0.01	20.67 ± 0.01	13.17 ± 0.001	20.70 ± 0.02	13.40 ± 0.005	22.00 ± 0.67

* Values are represented as the mean ± S.D. of three experiments; BS = Barishal sample, DS = Dinajpur sample, MS = Mymensingh sample, RS = Rajshahi sample, and PS = Pabna sample.

Table 4. Antifungal activity of hog plum (*Spondias mombin* L.) peel oil extracted from different regions of Bangladesh.

SN	Fungal Strains	Diameter of Zone of Inhibition (mm) *									
		BS		DS		MS		RS		PS	
		Cultures (5 µL/Petridish)	Standard (Fluconozole) (10 µg/disc)	Cultures (5 µL/Petridish)	Standard (Fluconozole) (10 µg/disc)	Cultures (5 µL/Petridish)	Standard (Fluconozole) (10 µg/disc)	Cultures (5 µL/Petridish)	Standard (Fluconozole) (10 µg/disc)	Cultures (5 µL/Petridish)	Standard (Fluconozole) (10 µg/disc)
1	*A. niger*	11.33 ± 0.17	19.13 ± 0.35	11.27 ± 0.02	19.43 ± 0.01	11.07 ± 0.0002	18.90 ± 0.0002	11.63 ± 0.0003	18.9 ± 0.020	11.10 ± 0.0005	19.73 ± 0.0005
2	*P. oxalicum*	13.17 ± 0.17	20.15 ± 0.77	12.93 ± 0.02	20.15 ± 0.77	12.87 ± 0.0004	19.87 ± 0.004	12.93 ± 0.002	19.3 ± 0.003	13.67 ± 1.97	20.37 ± 0.001

* Values are represented as the mean ± S.D. of three experiments; BS = Barishal sample, DS = Dinajpur sample, MS = Mymensingh sample, RS = Rajshahi sample, and PS = Pabna sample.

4. Conclusions

This study showed that the peel of *S. mombin* appears to be unique in terms of its volatile composition. The results indicate that the essential oils of different regions showed varying anti-microbial activities against the various food-borne pathogenic bacteria and fungi tested. The highest zone of inhibition was shown against *Staphylococcus aureus* and *Penicillium oxalicum*. On the other hand, the highest number of VCs was identified in peel samples from the Barishal district, whereas the lowest number was in the Dinajpur district samples. On the contrary, the highest number of VCs was found in the Dinajpur district samples, whereas the lowest number was in the Rajshahi district samples. However, the variation in the number of VCs of the samples was very small. Thus, *S. mombin* could become an alternative to synthetic bactericides and fungicides for use in agro-industries. This study provides data for the development of a more economical, useful, and eco-friendly bio-alternative to existing usages of hog plum peels. However, further studies on the safety and toxicity of these oils and their possible in vivo bioactivity should be carried out before use.

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Phenolic Compounds and Antioxidant Properties of Field-Grown and In Vitro Leaves, and Calluses in Blackberry and Blueberry

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Abstract: The aim of this study was to evaluate the content and profile of the phenolic compounds (PCs) and antioxidant properties of field-grown leaves, in vitro leaves and in vitro callus cultures of the blackberry 'Čačanska Bestrna' and blueberry 'Toro'. In vitro shoots of the selected genotypes were grown either on original Murashige and Skoog (MS) medium containing 1 mg/L BA, 0.1 mg/L IBA and 0.1 mg/L GA_3 ('Čačanska Bestrna') or on MS medium with macroelements reduced to 1/2, 2 mg/L zeatin and 0.2 mg/L IAA ('Toro'). Callus cultures were induced from in vitro leaves and established on MS medium with 2 mg/L BA and 2 mg/L 2,4-D ('Čačanska Bestrna') or MS medium with half strength macroelements, 2 mg/L BA, 2 mg/L 2,4-D and 1 mg/L NAA ('Toro'). Total phenolic (TPC) and flavonoid content (TFC) were the highest in blueberry leaves, whereas low TPC and TFC values were obtained in callus cultures of both cultivars. A higher content of PCs in blueberry leaves compared to blackberry leaves was determined by the UHPLC-DAD MS/MS technique. Quercetin derivatives and phenolic acids were the dominant PCs in the leaves of both berries, whereas gallocatechin was present in a significant amount in blueberry leaves. Callus cultures of both berries had a specific PC profile, with none detected in the leaves except quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside. Blackberry leaves showed the best antioxidant properties as estimated by ferric reducing power (FRP), $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity assays. Callus cultures of both berries exhibited three to five times lower $ABTS^{\bullet+}$ and ten to seventeen times lower $DPPH^{\bullet}$ scavenging activity compared to corresponding leaves. The analyzed leaves and callus cultures can be a good source of PCs with good antioxidant properties and specific phenolics, respectively, for applications in the food and pharmaceutical industries.

Keywords: open-field plants; tissue culture plants; in vitro callus culture; *Rubus* subg. *Rubus* Watson; *Vaccinium corymbosum* L.; phenolics; radical scavenging activities; ferric reducing power

1. Introduction

Blueberry and blackberry have high economic importance, particularly in hilly and mountainous regions of Serbia. The special economic importance of these fruit species is determined by the high usage value of their fruits, the profitability of production, high margins, contribution to additional employment, and more [1]. Furthermore, the small edible and colored berries of genera *Vaccinium* and *Rubus* are recognized as a good source of bioactive compounds (BCs), primarily phenolics, which contribute to the berries' organoleptic properties and after consumption exert a positive impact on human health [2,3]. In addition to their highly valuable fruits, the leaves of berry plants are also a rich source of phenolics and are often used in traditional medicine to treat numerous diseases such as colds, various inflammations, diabetes [4]. Leaves are present as a byproduct of growing berries and can be used as an alternative source of bioactive compounds which can be applied further for the development of functional food products and nutraceuticals [4–6]. Moreover, the European Medicines Agency (EMA) has approved the marketing and use of some *Rubus* leaf extracts and infusions for medical purposes [4]. Due to the mentioned characteristics of both fruits and leaves there has been increasing interest in growing berries in recent years, with a special emphasis on blueberries and blackberries. Furthermore, there is great interest in evaluating the BC composition of berry leaves, in vitro screening of their antioxidant and antimicrobial properties, and evaluation of in vivo biological activities and conduction of clinical trials [5–11]. For example, the highest values of antioxidant properties evaluated with DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging or FRAP assays were 586.6 μmol TEAC/g DW, 862.4 μmol TEAC/g DW and 2674 μmol FEAC/g DW for 'Nanjin' variety among the 73 investigated blueberry cultivars [11]; that is, 45.0, 257, and 139 mg/g DW for leaves of 'Bluerain' and 'Vernon' varieties among 104 selected blueberry cultivars [12], respectively. On the other hand, some wild blackberry cultivars showed high ABTS$^{\bullet+}$ scavenging activity (212.69 mmol TE/g dm) and FRAP (192.91 mmol TE/g dm), as well as high correlations with total phenolic content and content of ellagitannins [9]. Leaf extracts of six commercial blueberry varieties [6] and *Vaccinium corymbosum* variety [13] showed good antimicrobial properties (MIC and MBC) against some gram negative and gram-positive bacteria strains. Thus far, the phenolic profiles, antioxidant and antimicrobial properties of field-grown leaves of different blueberry and blackberry cultivars, in particular, 'Toro' [6,11] and 'Čačanska Bestrna' [5], have been successfully evaluated. More precisely, the DPPH$^{\bullet}$ scavenging activity of blackberry ('Čačanska Bestrna') and blueberry ('Toro') leaves were 83.77% [5] and 305.0 μmol TEAC/g DW [11]. In addition, extracts of the leaves of Toro blueberry cultivar have shown good antibacterial potential against some bacterial strains such as *Staphylococcus aureus*, *Rhodococcus equi*, *K. pneumonia* and *E. fecalis* [6]. Furthermore, the extract of 'Toro' blueberry showed a good antimutagenic effect against different *Salmonella typhimurium* strains, with percent inhibitions of 32.98% (TA98) and 38.68% (TA100), which gives them the possibility of potential application as a safe and useful alternative for the prevention of mutations [6].

However, the use of highly valuable leaves from field-grown berry plants is often limited due to the frequent use of pesticides [14], as well as their seasonal availability. This is why there is growing interest in and questions about the potential propagation and growing of berry plants in vitro as a source of secondary metabolites, or the induction of callus cultures using a specific nutrient medium for targeted production of some BCs which have significant potential as antioxidants [15–18].

Several methods are available in plant tissue culture, among which organogenesis and callogenesis are the most commonly used [15]. Organogenesis involves the production of plant organs (shoots or roots), directly from meristems or indirectly from dedifferentiated cells which are known as callus [15]. The obtained in vitro plant cultures can be used as a sustainable and alternative source of valuable BCs, primarily phenolic compounds, which have potential application as food additives [17]. Some blueberry and blackberry cultivars have been successfully micropropagated via axillary shoot culture and through indirect shoot organogenesis [19–23]. However, according to our knowledge, the pheno-

lic profile and antioxidant properties of blueberry and blackberry leaves obtained from in vitro shoots grown on specific nutrient medium have not yet been examined. On the other hand, callogenesis creates an amorphous cell mass in response to the exposure of explants to various biotic and abiotic elicitors which further initiate or enhance the biosynthesis of specific BCs [15,24]. The produced calluses can be used for plant regeneration or for targeted production of important metabolites in the cell suspension [15,16,18]. The biosynthesis of phenolic compounds in plant calluses depends on numerous factors such as nutrient media, plant growth regulators (PGRs), precursor feeding and elicitors [17]. Plant callus/cell cultures have the ability to accumulate secondary metabolites, which is a very promising system for biotechnological production of specific phenolic compounds [16]. For example, callus culture of *Vitis vinifera* has shown promising potential for the production of phenolic compounds [25] or specific phenolic classes such as anthocyanins [26], resveratrol [27] or stilbenes [28]. Moreover, phenolic compounds from the callus culture of different grape varieties have been successfully used to enrich food products such as yogurt [29]. Furthermore, callus cultures of different blueberry [30,31], and blackberry [20] cultivars have already been successfully created. The antioxidant properties and total phenolic content of blackberry calluses [20], that is, the phenolic profile of some varieties of blackberry calluses [31], were determined, as a prerequisite for further biotechnological production of highly valuable active compounds. However, according to our knowledge, calluses of blueberry 'Toro' and blackberry 'Čačanska Bestrna' have not yet been produced and analyzed. Furthermore, the antioxidant properties of in vitro leaves and calluses of these two berry cultivars were not determined by FRP, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity assays until now.

Therefore, the aim of this study was to evaluate and to compare the content and profile of phenolic compounds of field-grown and in vitro leaves and callus cultures of blackberry 'Čačanska Bestrna' and blueberry 'Toro', as well as their antioxidant properties, using three common antioxidant assays: FRP, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities. Bearing in mind a number of advantages of the plant tissue culture-based production of secondary metabolites in comparison with conventional agricultural production, including controlled production via standardized protocols independent from seasonal variation, low water and carbon input, no use of pesticide and herbicides, etc. [32], and given the economic importance of these fruit species and their potential for targeted BC production, the results could help to estimate their possible use in the food and pharmaceutical industry.

2. Materials and Methods

2.1. Plant Materials

Research was conducted using blackberry cultivar 'Čačanska Bestrna' (*Rubus* subg. *Rubus* Watson) and blueberry cultivar 'Toro' (*Vaccinium corymbosum* L.). 'Čačanska Bestrna' is a cultivar developed at the Fruit Research Institute, Čačak, which has been widely planted in Serbia; it displays excellent performance regarding cropping, fruit quality and resistance to diseases. In the group of semi-erect thornless blackberry cultivars, this cultivar reaches about 10% of the total world production. Although not widely grown in Serbia, 'Toro' belongs to the most common mid-season highbush blueberry cultivars in Central and Eastern European countries [33] and represents a self-fertile and heavy producer with large, juicy, sweet, and never tart berries. In addition to berries, the leaves of both cultivars appear to be good sources of antioxidants and have strong antibacterial activity [4,5].

Field-grown leaves as well as in vitro leaves and callus cultures of the two berry plant genotypes were obtained from the Fruit Research Institute, Čačak, Serbia. In vitro shoots and calluses were cultivated on nutrient media in a growth room at 23 ± 1 °C, under 16 h-photoperiod and light intensity of 8.83 W/m^2, using white fluorescent tubes (6500 K, 40 W) (Tissue Culture Laboratory of Fruit Research Institute), while field-grown leaves were obtained from plants grown in the Institute's research fields.

2.2. In Vitro Shoot Cultivation and Callus Induction from Leaves

Field grown plants of both cultivars were used as the source of initial explants for in vitro culture. Aseptic culture was established using single-node cuttings of newly formed shoots taken from branches during the spring. Cuttings with axillary buds were submerged in lukewarm water with a few drops of Tween 20 for 30 min and washed under running tap water for 2 h, followed by sterilization in 70% ethanol (1 min) and in a solution containing 0.1% $HgCl_2$ and 0.01% Tween 20 (5 min), and finally washed with sterile distilled water (3 × 5 min) to remove all traces of disinfectants.

After establishment of aseptic cultures, shoots of examined genotypes were grown and multiplied on original or partially modified Murashige and Skoog (MS) medium [34], (Table S1). The axillary shoot proliferation of blackberry 'Čačanska Bestrna' and blueberry 'Toro' are presented in Figures S1 and S2. Composition of the media used for in vitro shoot multiplication of berry plant genotypes are listed in Table 1.

Table 1. Composition of media used for micropropagation of blackberry and blueberry.

Genotype	Macro-Elements	Micro-Elements	Vitam.	Plant Growth Regulators (PGRs)	Sucrose	Agar	pH
Blackberry 'Čačanska Bestrna'	MS	MS	MS	6-Benzylaminopurine (1 mg/L) Indole-3-butyric acid (0.1 mg/L) Gibberellic acid (0.1 mg/L)	20 g/L	7 g/L	5.7–5.8
Blueberry 'Toro'	MS 1/2	MS	MS	Zeatin (2 mg/L) Indole-3-acetic acid (0.2 mg/L)	20 g/L	7 g/L	4.5

MS—Murashige and Skoog basal medium.

Both blackberry and blueberry callus cultures were induced from in vitro leaves. Leaves were collected from the upper third of in vitro propagated shoots, cut three times transversely across the mid-vein and placed with the adaxial surface touching regeneration medium poured into Petri dishes (9 cm in diameter, around 50 mL of medium). The ingredients of media used for induction and maintaining of callus cultures are presented in Table 2.

Table 2. Composition of media used for callus induction.

Genotype	Macro-Elements	Micro-Elements	Vitam.	Plant Growth Regulators (PGRs)	Sucrose	Agar	pH
Blackberry 'Čačanska Bestrna'	MS	MS	MS	6-Benzylaminopurine (2 mg/L) 2,4-dichlorophenoxyacetic acid (2 mg/L)	30 g/L	7 g/L	5.7–5.8
Blueberry 'Toro'	MS 1/2	MS	MS	6-Benzylaminopurine (2 mg/L) 2,4-dichlorophenoxyacetic acid (2 mg/L) α-Naphthaleneacetic acid (1 mg/L)	30 g/L	7 g/L	4.5

MS—Murashige and Skoog basal medium.

In vitro shoot and callus cultures of blackberry 'Čačanska Bestrna' and blueberry 'Toro' are presented in Figure 1.

Field-grown and in vitro young and newly formed leaves of berry plants as well as their callus cultures were used for further phenolic characterization and the evaluation of antioxidant properties.

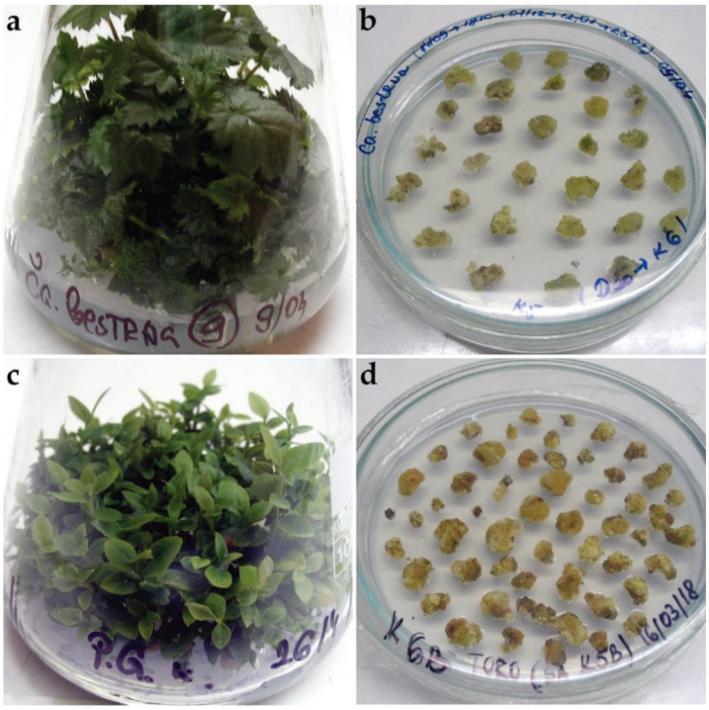

Figure 1. In vitro shoot and callus cultures of blackberry 'Čačanska Bestrna' (**a**,**b**) and blueberry 'Toro' (**c**,**d**).

2.3. Preparation of Leaves and Callus Culture Extracts

Collected samples of leaves and calluses were finely ground and homogenized with liquid nitrogen using an Ika A11 basic mill. The extracts were then prepared using the extraction protocol previously described by Pavlović et al. [35], with slight modifications. Briefly, previously ground samples (1 g) were extracted using 80% methanol with 0.1% HCl on a mechanical stirrer (Mechanical stirrer Thys 2) for 1 h. After that, the samples were centrifuged for 10 min at 4000× *g* (Janetzki T32c, Wallhausen, Germany) and filtered through Whatman No. 1 filter paper. The extraction procedure was repeated twice and supernatants were collected. Furthermore, combined supernatants were evaporated using a rotary evaporator to dryness (40 °C) (Laborota 4000, Heidolph Instruments, Schwabach, Germany) and reconstituted in 10 mL miliQ water for further analysis. The suspensions were filtrated through 0.45 µm syringe filters before further spectrophotometric and UHPLC-DAD MS/MS analysis. These extracts represented aqueous extracts of samples.

2.4. Total Phenolic and Flavonoid Content

Total phenolic and flavonoid content in the blueberry/blackberry leaves and calluses were determined using a colorimetric assay with Folin-Ciocalteu's reagent [36]; that is, assay with aluminium chloride [37]. Absorbance was measured at 765 nm for TPC and 510 nm for TFC, using a Shimadzu UV-1800 spectrophotometer (Shimadzu USA Manufacturing, Inc., Canby, OR, USA). Results for TPC were expressed in mg of gallic acid equivalent (mg GAE/g DW), while results for TFC were expressed as catechin equivalents (mg CE/g DW), both per g dry weight of samples.

2.5. UHPLC-DAD MS/MS Analysis of Leaves and Calluses

The identification and quantification of phenolic compounds in leaves and calluses of 'Toro' and 'Čačanska Bestrna' was conducted using a Dionex Ultimate 3000 UHPLC system equipped with diode array detector (DAD) and TSQ Quantum Access Max triple-quadrupole mass spectrometer (MS) (ThermoFisher Scientific, Basel, Switzerland), as previously detailed by Pešić et al. [37]. A Syncronis C18 column (100 × 2.1 mm, 1.7 mm particle size) from Thermo Fisher Scientific was used as the analytical column for separation. The mass spectrometry data were acquired in the negative ion mode, in the m/z range from 100 to 1000. Full scanning and product ion scanning (PIS) were conducted for the qualitative analysis of the targeted phenolic compounds. The collision-induced dissociation experiments were performed using argon as the collision gas, and the collision energy varied depending on the compound. The time-selected reaction monitoring experiments for quantitative analysis were performed using two MS^2 fragments for each compound that was previously defined as dominant in the PIS experiments (Table S2). Other chromatographic and MS settings were the same as in Pešić et al. [37]. However, it should be noted that in this study only MS data of commercially available standards were used for both identification and quantification of PCs. The Xcalibur software (version 2.2) was used for instrument control as well as for the acquisition and analysis of data. Calculation of the concentrations was based on the external standard method. Standards of phenolic compounds (gallic acid, vanillic acid, ferulic acid, syringic acid, chlorogenic acid, catechin, catechin gallate, gallocatechin, quercetin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, isohramnetin-3-*O*-rutinoside, isohramnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, kaempferol, apigenin-7-*O*-glucoside, naringenin, aesculetin, and phlorizin) were obtained from Sigma Aldrich (Steinheim, Germany). The total amounts of each identified compound were evaluated via calculation of the peak areas and expressed as mg/kg dry weight (DW) of the sample.

2.6. Antioxidant Properties

2.6.1. Ferric Reducing Power (FRP)

The ferric reducing power of leaves and callus extracts was determined according to the method previously described by Pešić et al. [37], with slight modifications. Briefly, an aliquot of the appropriately diluted sample (2.5 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH = 6.6) and 2.5 mL of 1% potassium ferricyanide. Then, the homogenized mixture was incubated at 50 °C, and after 20 min it was mixed with 2.5 mL of 10% TCA. Supernatant collected after centrifugation (2.5 mL) was mixed with 2.5 mL of milliQ water and 0.5 mL of 0.1% ferric chloride. After 10 min, the absorbance of the mixture was measured at 700 nm. Results for FRP were expressed as mg gallic acid equivalents per g dry weight of the sample.

2.6.2. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ Radical Scavenging Activity

$ABTS^{\bullet+}$ scavenging activity was evaluated as previously reported by Pešić et al. [37]. Prepared $ABTS^{\bullet\pm}$ working solution (1 mL) was mixed with the sample (10 µL) and left in the dark for 7 min. Afterwards, the absorbance of the mixture was measured at 734 nm.

$DPPH^{\bullet}$ scavenging activity was performed according to a method previously reported by Gawron-Gzella et al. [38], with small modifications. Briefly, 120 µL of prepared 150 µM $DPPH^{\bullet}$ working solution was mixed with 15 µL of the sample and incubated for 30 min in the dark. Next, the absorbance was measured at 515 nm using a Plate Reader (Tecan Sunrise Spectrophotometer, Tecan Trading AG, Switzerland).

The percentage of $ABTS^{\bullet\pm}$ and $DPPH^{\bullet}$ quenched radicals was calculated using the following Equation (1):

$$ABTS^{\bullet+}/DPPH^{\bullet} \text{ radical scavenging activity (\%)} = [(Ac - As)/Ac] \times 100 \quad (1)$$

where Ac is control absorbance (ABTS$^{\bullet\pm}$ or DPPH$^{\bullet}$ working solution mixed with 10 µL of methanol) and As is absorbance of the sample mixed with ABTS$^{\bullet+}$ or DPPH$^{\bullet}$ working solution.

The ABTS$^{\bullet+}$ scavenging activity was expressed as µg of ascorbic acid equivalents per mL of sample (µg AAE/mL).

2.7. Statistical Analysis

All of the results were performed in triplicate and presented as the mean values ± standard deviation (SD). Data for TPC, TFC and antioxidant properties were analyzed using Two-way ANOVA considering the origin of the samples (field-grown leaves, in vitro leaves and callus cultures) and berry plant cultivars as fixed effects and the replicates as a random effect. Significant differences ($p < 0.05$) between means were determined by Tukey's test, using GraphPad Prism6 software (USA). Significant differences ($p < 0.05$) between the means for individual phenolics were determined by Student's *t*-test. The correlation analyses were performed by calculating Pearson's correlation coefficient (r), ($p < 0.05$), using the Statistica software version 12.0 (StatSoft Co., Tulsa, OK, USA). Figures were drawn using GraphPad Prism6 software (USA).

3. Results and Discussion

3.1. Total Phenolic and Flavonoid Content

The TPC of aqueous extracts of blueberry and blackberry leaves and calluses are presented in Figure 2a.

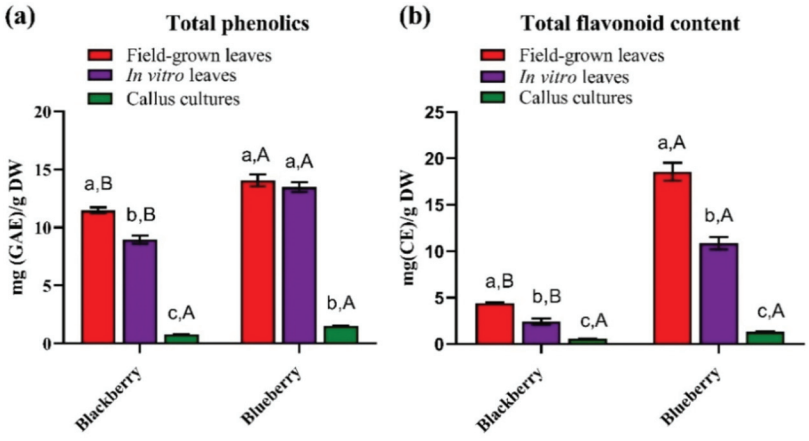

Figure 2. (**a**) Total phenolic content and (**b**) Total flavonoid content of blackberry 'Čačanska Bestrna' and blueberry 'Toro' leaves and callus samples. The bars with (±) standard deviation represent mean values. The different lowercase letters indicate a significant difference ($p < 0.05$) between field-grown leaves, in vitro leaves and callus culture of the same berry; different uppercase letters indicate a significant difference ($p < 0.05$) between the same type of samples of two different berry cultivars.

The TPC of aqueous extracts of field-grown and in vitro leaves of 'Toro' blueberry were 14.06 ± 0.51 and 13.47 ± 0.42 mg GAE/g DW, without a statistically significant difference ($p < 0.05$). These values are significantly lower (almost 10-fold lower) than the TPC values for field-grown leaves of six cultivated blueberry cultivars, including 'Toro' (132.92 mg GAE/g leaves material), reported by Ștefănescu et al. [6]. In addition, Wu et al. [11] also reported significantly higher TPC values for blueberry field-grown leaves from 73 different cultivars collected in southern China, which ranged from 32.18 ± 0.01 ('O'Neal') to 224.1 ± 3.4 ('Blackpearl') mg GAE/g DW. Moreover, the same study determined TPC values in the leaves of field-grown 'Toro' plants of 75.07 ± 1.48 mg GAE/g

DW, which is 5.3- and 5.6-fold higher than the TPC values obtained in this study for both field-grown and in vitro leaves for the same cultivar, respectively. Goyali et al. [39] also obtained higher TPC values for the leaves of wild lowbush blueberry clone QB9C (*Vaccinium angustifolium* Ait.) originating from both ex vitro (propagation by stem cuttings) and in vitro propagated plants. These differences are mainly due to the differences in extract solvents. Namely, in this study the methanol extracts of leaves and calluses were evaporated, resuspended in milliQ water and, after filtration through 0.45 µm syringe filters, subjected to spectrophotometric analysis, whereas the aforementioned authors used 40% ethanol, 85% methanol or 80% acetone. Furthermore, differences in method of extraction (solid-liquid against ultrasound-assisted extraction), applied in vitro propagation methods as well as the different geographical areas where the berry plant is grown, climatic factors and soil composition for field-grown plants can also affect the TPC value of analyzed samples [4,5,36]. Furthermore, total phenolics for both aqueous extracts, field-grown and in vitro leaves of 'Toro' blueberry were significantly higher ($p < 0.05$), in comparison to those of field-grown and in vitro leaves of 'Čačanska Bestrna', whose TPC values were 11.46 ± 0.27 and 8.97 ± 0.35 mg GAE/g DW, respectively. These values are significantly lower than previously reported TPC value for field-grown leaves of the same blackberry cultivar, also collected in Serbia [5]. However, the TPC value of field-grown blackberry leaves obtained in this study was significantly higher than the TPC values obtained for water extracts from the leaves of wild-grown and cultivated blackberry (*Rubus fruticosus* L. 'Thornfree') collected during different seasons of the year [40]. In the study conducted by Fathy et al. [20], TPC values for methanolic extracts obtained from the leaves of in vitro blackberry (*Rubus fruticosus* L.) shoots grown on media with different concentrations of PGRs (benzyladenine (BA) at different concentrations, applied alone or in combination with α-naphthaleneacetic acid (NAA)), were in the range from 1.17 to 2.39 mg GAE/g, which is significantly less than the TPC value obtained for in vitro blackberry leaves in this study. Thus, PGR combinations applied to in vitro cultivation of blackberry 'Čačanska Bestrna' should be considered as promising, because in vitro leaves can be a good source of phenolic compounds for further applications in the food industry and pharmacy. As can be seen in Figure 2a, callus culture induced from both berry plants had significantly lower TPC in comparison with their leaves; the values were 1.52 ± 0.03 and 0.78 ± 0.03 mg GAE/g DW in blueberry 'Toro' and blackberry 'Čačanska Bestrna', respectively. The TPC value of blueberry callus culture was significantly lower than those reported by Ramata-Stunda et al. [31], and significantly higher than TPC values reported by Ghosh et al. [30] in callus culture of different blueberry cultivars. The TPC value of blackberry callus culture in this study was in agreement with the TPC values obtained for callus culture induced from blackberry (*Rubus fruticosus* L.) leaves treated with different concentrations of NAA and/or 2,4-dichlorophenoxyacetic acid (2,4-D) [20].

The total flavonoid content of aqueous extracts of 'Toro' and 'Čačanska Bestrna' leaves and their callus cultures are illustrated in Figure 2b. TFC values of aqueous extracts of field-grown and in vitro blueberry leaves were 18.56 ± 0.98 and 10.88 ± 0.66 mg CE/g DW, respectively. On the other hand, the TFC values of aqueous extracts of field-grown and in vitro leaves in blackberry were significantly lower (almost five-fold) in comparison with corresponding blueberry samples, i.e., 4.42 ± 0.11 and 2.44 ± 0.34 mg CE/g DW, respectively. Interestingly, the TFC value in the extract of field-grown blueberry leaves was significantly higher in comparison to the TPC value of the same extract, which was also observed in the study reported by Wu et al. [11]. TFC values in field-grown leaves of blueberry 'Toro' previously reported by Ștefănescu et al. [6] and Wu et al. [11], showed significantly higher flavonoid content in comparison to the value obtained in this study. As shown in Figure 2b, callus culture for both berry plants had significantly lower TFC values in comparison to the results obtained for their leaves, which is in accordance with the obtained results for TPC. The TFC values of blueberry and blackberry callus cultures (1.31 ± 0.03 and 0.57 ± 0.01 mg CE/g DW, respectively) were not significantly different ($p < 0.05$). The literature survey led us to the conclusion that the data on the TFC

of blueberry and blackberry callus culture are rather scarce. Ghosh et al. [30] obtained considerably higher TFC values for the callus culture of different blueberry cultivars, in comparison with TFC value obtained for 'Toro' blueberry callus culture in this study. On the other hand, Fathy et al. [20] obtained very low TFC values for methanolic extract of the callus culture induced from blackberry leaves which were treated with different concentrations and combinations of PGRs, such as 2,4-D and NAA.

3.2. UHPLC-DAD MS/MS Analysis of Blueberry and Blackberry Leaves and Callus Cultures

Characterization and quantification of phenolic compounds from aqueous extracts of blueberry and blackberry leaves, as well as their callus cultures, were performed using UHPLC-DAD MS/MS analyzer (Table 3).

Depending on the berry plant genotype and specific characteristics of samples tested, a total of 20 phenolic compounds was confirmed and quantified. As can be observed from Table 3, field-grown and in vitro leaves of blueberry 'Toro' are better source of phenolic compounds in comparison to field-grown and in vitro leaves of blackberry 'Čačanska Bestrna'. The most abundant PCs of 'Toro' leaves belong to phenolic acids, flavonols and flavan-3-ols, with respective shares of 41.3, 54.3, and 4.3% for field-grown, and 29.7, 54.6, and 14.9% for in vitro leaves. The dominant presence of phenolic acids and flavonols has been shown in some previous PC characterizations of leaves of different blueberry cultivars using the chromatographic technique [6,10–12]. Quercetin derivatives, chlorogenic acid and gallocatechin were dominant the PCs for both field-grown and in vitro blueberry leaves. Other studies have also reported quercetin derivatives [6,11] and chlorogenic acid [4,12,31] as the most abundant PCs in the leaves of various blueberry cultivars, including 'Toro'. Among quercetin derivatives, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside were the most predominant, with respective contents of 160.113 ± 3.059 and 49.639 ± 1.526 mg/kg DW for extracts of field-grown leaves and 115.598 ± 5.041 and 62.293 ± 4.403 mg/kg DW for extracts of in vitro leaves. This is in agreement with the result reported by Ștefănescu et al. [6] for field-grown leaf extract of various blueberry cultivars, including 'Toro'. Interestingly, significant amounts of flavonols, such as quercetin-3-*O*-rhamnoside, aesculetin, and the aglycones of quercetin and kaempferol, have been identified only in extracts of in vitro blueberry leaves.

On the other hand, the quercetin derivatives syringic and chlorogenic acid were the most abundant phenolic compounds detected in field-grown and in vitro leaves of blackberry 'Čačanska Bestrna', making more than 95% of all quantified phenolics. Some previous studies have reported significantly higher contents of different phenolic acids and flavonoids in methanolic extracts of field-grown leaves of three blackberry wild genotypes and three cultivars [7], or 26 different wild blackberry genotypes collected from various localities throughout Poland [9]. Flavan-3-ols were not detected in the leaves of 'Čačanska Bestrna', which is not in accordance with the results reported by Pavlović et al. [5], which found significant amounts of catechin derivatives in methanolic extract of field-grown leaves of the same blackberry cultivar, also collected in Serbia. The absence of flavan-3-ols can be attributed to their ability to rapidly polymerize in aqueous extract into complex forms, which were removed by filtration through a 0.45 µm filter before UHPLC-DAD MS/MS analysis. Differences in the phenolic profiles of leaves originating from in vitro cultivated plants may be due to applied PGRs [17] such as zeatin and indol-3-acetic acid for blueberry 'Toro'; that is, gibberellic acid (GA_3), BA and indole-3-butyric acid (IBA) for blackberry 'Čačanska Bestrna' (Table 1).

Table 3. The content of phenolic compounds in field-grown and in vitro leaves, as well as in callus cultures of blueberry and blackberry.

Samples	Blueberry 'Toro'			Blackberry 'Čačanska Bestrna'		
	Field-Grown Leaves	In Vitro Leaves	Callus Culture	Field-Grown Leaves	In Vitro Leaves	Callus Culture
Phenolics (mg/kg)						
			Phenolic acids			
Gallic acid	/	/	/	/	/	0.202 ± 0.005
Vanillic acid	/	/	0.136 ± 0.009	/	/	/
Ferulic acid	/	/	0.330 ± 0.021 ^{b}	/	/	0.154 ± 0.017 ^{c}
Syringic acid	/	/	/	11.348 ± 1.125 ^{a}	6.701 ± 0.243 ^{b}	/
Chlorogenic acid	162.817 ± 11.251 ^{b}	111.826 ± 3.174 ^{c}	/	9.484 ± 0.591 ^{d}	2.922 ± 0.041 ^{e}	/
Sum	**162.817 (41.3)**	**111.826 (29.7)**	**0.466**	**20.832 (56.4)**	**9.623 (73.6)**	**0.356**
			Flavan-3-ols			
Catechin	/	/	0.567 ± 0.045 ^{b}	/	/	/
Catechin gallate	/	/	0.051 ± 0.004 ^{b}	/	/	0.048 ± 0.002 ^{b}
Gallocatechin	17.077 ± 1.078 ^{a}	56.239 ± 2.816 ^{b}	/	/	/	/
Sum	**17.077 (4.3)**	**56.239 (14.9)**	**0.618**	**/**	**/**	**0.048**
			Flavonols			
Quercetin	/	17.304 ± 1.315	/	/	/	/
Quercetin-3-*O*-rutinoside	49.639 ± 1.526 ^{c}	62.293 ± 4.403 ^{d}	0.085 ± 0.006 ^{b}	7.395 ± 0.197 ^{e}	1.061 ± 0.079 ^{f}	/
Quercetin-3-*O*-glucoside	160.113 ± 3.059 ^{c}	115.598 ± 5.041 ^{d}	0.390 ± 0.016 ^{e}	5.483 ± 0.479 ^{f}	1.697 ± 0.239 ^{g}	0.183 ± 0.007 ^{h}
Quercetin-3-*O*-rhamnoside	/	6.533 ± 0.528	/	/	/	/
Isohramnetin-3-*O*-rutinoside	/	/	0.034 ± 0.001	/	/	/
Isohramnetin-3-*O*-glucoside	/	/	0.093 ± 0.003	/	/	/
Kaempferol-3-*O*-glucoside	4.538 ± 0.207 ^{b}	2.598 ± 0.249 ^{c}	/	2.540 ± 0.100 ^{c}	0.154 ± 0.015 ^{d}	/
Kaempferol	/	1.435 ± 0.036	/	/	/	/
Apigenin-7-*O*-glucoside	/	/	/	0.284 ± 0.027	/	/
Sum	**214.29 (54.3)**	**205.761 (54.6)**	**0.601**	**15.702 (42.3)**	**2.912 (22.3)**	**0.183**
			Other detected phenols			
Naringenin	0.361 ± 0.028	/	/	/	/	/
Aesculetin	/	2.848 ± 0.181 ^{a}	/	0.432 ± 0.024 ^{b}	0.530 ± 0.028 ^{c}	/
Phlorizin	/	/	0.068 ± 0.003 ^{a}	/	/	0.058 ± 0.004 ^{b}
Sum	**0.361**	**2.848**	**0.068**	**0.432**	**0.530**	**0.058**
Total	**394.545**	**376.674**	**1.753**	**36.965**	**13.065**	**0.645**

Means with the same letter in the same row are not significantly different, *t*-test ($p < 0.05$), (mean ± S.D.; n = 3); /—not detected. Values in parentheses represent a relative amount of phenolic class in the field-grown and in vitro leaves for both berry plant cultivars.

Generally, the field-grown and in vitro blueberry/blackberry leaves have shown to be a much better source of phenolic compounds (PCs) in comparison to in vitro induced callus culture obtained from in vitro leaves of the same berry cultivars. The low yield of phenolic compounds in the callus culture of blueberry 'Toro' and blackberry 'Čačanska Bestrna' can be explained by the lack of cell differentiation [15]. The callus culture of 'Toro' produced higher levels of specific phenolic acids, flavan-3-ols and flavonols, in comparison with the callus culture of 'Čačanska Bestrna'. However, both aqueous extract of blueberry and blackberry callus cultures contained small amounts of individually detected PCs. Interestingly, except for quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside, other identified phenolics are specific for blueberry callus culture and have not been detected in field-grown and in vitro blueberry leaf extracts. For example, small amounts of phenolic acid such as vanillic and ferulic acid (0.330 ± 0.021 mg/kg DW), or flavan-3-ols such as catechin (0.567 ± 0.045 mg/kg DW) and catechingallate, were found in the callus extract of 'Toro', while chlorogenic acid and gallocatechin were not detected. However, in the study of Ramata-Stunda et al. [31], chlorogenic acid was the most abundant PC in the callus culture of 'Duke' and 'Bluecrop' blueberries, while other phenolic acids were present in traces. This may be due to the presence of phytohormones and elicitors in nutrient medium, which are able to initiate the synthesis of specific PCs [16,17]. On the other hand, although in small amounts, quercetin-3-*O*-glucoside, gallic and ferulic acid were found as dominant PCs in the callus culture of blackberry 'Čačanska Bestrna', while other phenolics were detected in traces. Interestingly, the predominantly confirmed syringic and chlorogenic acids in field-grown and in vitro blackberry leaf extracts were not found in the callus culture of this berry plant, but gallic and ferulic acid were. However, the effect of different PGRs on the synthesis of specific PCs in the callus cultures of 'Toro' and 'Čačanska Bestrna' requires a more complex experiment and additional research.

3.3. Antioxidant Properties

Using different in vitro screening assays for evaluating the antioxidant properties of prepared aqueous extracts of field-grown leaves, in vitro leaves and callus cultures of blackberry and blueberry gives better insight into their potential opportunities for health improvement and further applications. In order to obtain more detailed information on the antioxidant properties of the analyzed samples, it is very important to apply several different tests based on different mechanisms of action. Three mechanisms of action of bioactive compounds are generally known: hydrogen atom transfer (HAT), single electron transfer (SET), and the ability to chelate transition metals [41–43]. These mechanisms include several tests most commonly used to assess the antioxidant properties of phenolics extracts of leaves and callus cultures of berry plants [11,12,20,31,44]. In this study, three in vitro screening antioxidant assays, FRP, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity, were used with the aim of evaluating the antioxidant potential of the leaves and calluses of blueberry and blackberry cultivars (Figure 3).

According to our knowledge, this is the first time the antioxidant properties of in vitro leaves and calluses of these berry plant cultivars has been evaluated by performing these three assays.

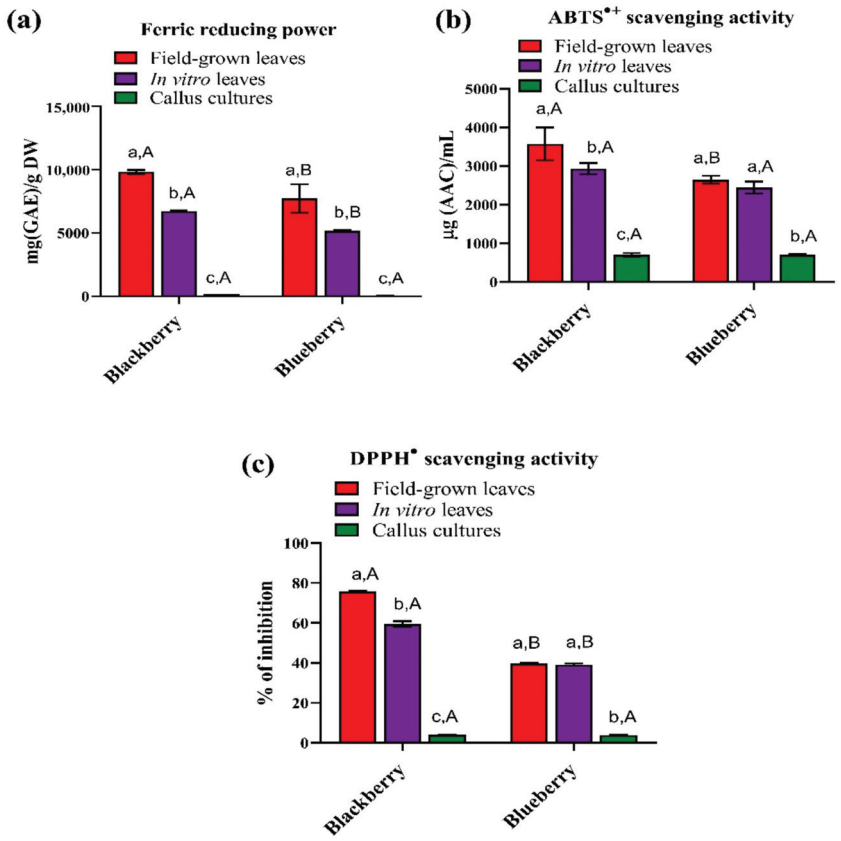

Figure 3. (**a**) Ferric reducing power (FRP), (**b**) ABTS$^{\bullet+}$ radical scavenging activity, and (**c**) DPPH$^{\bullet}$ radical scavenging activity of field-grown leaves, in vitro leaves and callus cultures of blackberry 'Čačanska Bestrna' and blueberry 'Toro'. The bars with (±) standard deviation represent mean values. The different lowercase letters indicate a significant difference ($p < 0.05$) between field-grown leaves, in vitro leaves and callus culture of the same berry. Different uppercase letters indicate a significant difference ($p < 0.05$) between the same types of samples of two different cultivars.

3.4. Ferric Reducing Power (FRP)

The Ferric reducing antioxidant power (FRAP) assay was most commonly used in the literature to assess the reducing properties of extracts of blueberry [11,12] and blackberry [9,40] leaves. However, this assay has some limitations, such as the low pH value applied and the reactivity rate of different types of molecules, giving variable results [42]. On the other hand, the ferric reducing power assay is based on the ability of bioactive compounds to reduce Fe^{3+}-ferricyanide complexes to their ferrous (Fe^{2+}) form in more relevant physiological conditions (pH = 6.6) [42], and can serve as a significant indicator of antioxidant activity of prepared leaves and callus culture extracts of blueberry and blackberry.

The aqueous extracts of field-grown and in vitro leaves of blueberry 'Toro' and blackberry 'Čačanska Bestrna' analyzed in this study showed good ferric reducing ability (Figure 3a). More precisely, the FRP values for field-grown leaves were 7722.75 ± 1125.62 ('Toro') and 9815.27 ± 155.52 ('Čačanska Bestrna') mg GAE/g DW, while for in vitro leaves the values were 5153.76 ± 88.37 and 6731.56 ± 50.96 mg GAE/g DW, respectively. As can be seen in Figure 3a, the FRP values of field-grown leaves for both berry plants leaves were significantly higher ($p < 0.05$) in comparison with the FRP values of in vitro leaves, which

is in agreement with TPC and TFC results. Good ferric reducing ability of field-grown blackberry and blueberry leaves has also been reported by other authors [9,11,12]. However, a direct comparison is not possible, primarily due to the previously mentioned FRAP method used by the authors to measure the reducing ability of leaf extracts. Interestingly, field-grown and in vitro leaves of blackberry 'Čačanska Bestrna' had better FRP activity than field-grown and in vitro leaves of blueberry 'Toro' (Figure 3a). This contrasts with the results obtained by chromatographic, TPC and TFC analysis, which showed a more diverse and higher content of phenolic compounds in the leaves of 'Toro'. This can be explained by the fact that field-grown and in vitro leaf extracts of blackberry contain other highly effective antioxidants in addition to the quantified phenolic compounds, such as ellagic acid derivatives, ellagitannins [5,9], vitamin C [45] and some terpenes [46] in significant quantities, which have not been analyzed in this study. On the other hand, aqueous extracts of callus cultures in both berry plants showed very low ferric reducing ability, which is consistent with the results obtained for their individual and total phenolic content. Correlation analysis revealed a significant positive correlation ($p < 0.05$) between FRP and TPC ($r = 0.87$).

3.5. ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ Scavenging Activity

The ABTS$^{\bullet+}$ scavenging activity of aqueous extracts of field-grown leaves of blackberry 'Čačanska Bestrna' was 3574.10 ± 426.78 μgAAC/mL, which was significantly higher than ABTS$^{\bullet+}$ values for aqueous extracts of in vitro leaves of the same cultivar (2933.51 ± 147.84 μgAAC/mL) or field-grown and in vitro leaves of blueberry 'Toro' (Figure 3b). ABTS$^{\bullet+}$ scavenging activity of aqueous extracts of field-grown and in vitro leaves of blueberry were 2644.22 ± 98.43 and 2442.74 ± 155.24 μgAAC/mL, without significant differences. As with the FRP results, aqueous extracts of field-grown and in vitro leaves of blackberry showed ABTS$^{\bullet+}$ values significantly higher than ABTS$^{\bullet+}$ values for aqueous extracts of field-grown and in vitro leaves of blueberry. This is not in accordance with the chromatographic and spectrophotometrically obtained results for individual and total phenolics, which are significantly higher for blueberry 'Toro'. A previous study by Pavlović et al. [5] using UHPLC-LTQ OrbiTrap MS/MS analysis showed that the leaves of 'Čačanska Bestrna' blackberry predominantly contain ellagic acid and its derivatives, as well as ellagitannins, which probably contribute to the high ABTS$^{\bullet+}$ values. Due to the large number of hydroxyl groups, ellagic acid and its derivatives, as well as ellagitannins, are potentially good hydrogen ion donors and scavengers of ABTS$^{\bullet+}$. Other authors have also noticed good ABTS$^{\bullet+}$ scavenging activity by different leaves of wild blackberry [9] and blueberry cultivars, including 'Toro' [11,12], but a direct comparison with our results is not possible due to differences in measurement units and the applied methods. The ABTS$^{\bullet+}$ scavenging activity of callus cultures were 701.48 ± 18.27 ('Toro') and 703.14 ± 46.50 ('Čačanska Bestrna') μgAAC/mL, without significant difference ($p < 0.05$) (Figure 3b). The obtained ABTS$^{\bullet+}$ values for callus cultures are about three-fold (blueberry), that is, about four-fold (blackberry) less than the ABTS$^{\bullet+}$ values obtained for field-grown and in vitro leaves of the same berry plants. To the best of our knowledge, this is the first time that the ABTS$^{\bullet+}$ scavenging activity of in vitro leaves and callus cultures of blueberry and blackberry has been determined.

The DPPH$^{\bullet}$ scavenging assay is most commonly used to evaluate the antioxidant properties of field-grown leaves [5,6,38,39,44] and callus cultures [30,31] of different blueberry and blackberry cultivars. In this study, similar to the FRP results, aqueous extracts of field-grown and in vitro leaves of blackberry 'Čačanska Bestrna' showed ABTS$^{\bullet+}$ values significantly higher than ABTS$^{\bullet+}$ values for aqueous extracts of field-grown and in vitro leaves of blueberry 'Toro'. This is not in compliance with the chromatographic and spectrophotometrically obtained results for individual and total phenolics, which are significantly higher for blueberry. A previous study by Pavlović et al. [5], using UHPLC-LTQ OrbiTrap MS/MS analysis showed that the leaves of 'Čačanska Bestrna' blackberry predominantly contain ellagic acid and its derivatives, as well as ellagitannins, which probably contribute to high

$ABTS^{\bullet+}$ values. Due to a large number of hydroxyl groups, ellagic acid, its derivatives, and ellagitannins are potentially good hydrogen ion donors and scavengers of $ABTS^{\bullet+}$. Other authors have also noticed good $ABTS^{\bullet+}$ scavenging activity of different leaves of wild blackberry [9] and blueberry cultivars, including 'Toro' [11,12], but a direct comparison with our results is not possible due to differences in measurement units and the applied methods. The $ABTS^{\bullet+}$ scavenging activity of callus cultures were 701.48 ± 18.27 ('Toro') and 703.14 ± 46.50 ('Čačanska Bestrna') μg AAC/mL, respectively, without significant differences ($p < 0.05$) (Figure 3b). The obtained $ABTS^{\bullet+}$ values for callus cultures are about three-fold (blueberry), that is, about five-fold (blackberry) less than the $ABTS^{\bullet+}$ values obtained for field-grown and in vitro leaves of the same berry plant. As far as we know, this is the first time that the $ABTS^{\bullet+}$ scavenging activity of in vitro leaves and callus cultures of blueberry and blackberry cultivars has been determined. Aqueous extracts of field-grown and in vitro leaves of blackberry 'Čačanska Bestrna' showed significantly higher percentage of inhibition of DPPH radicals (75.77% and 59.44%, respectively), than field grown (39.15%) and in vitro (39.65%) leaves of blueberry 'Toro' (Figure 3c). These $DPPH^{\bullet}$ results show the same trend as the results obtained for the $ABTS^{\bullet+}$ scavenging activity and FRP assays. Correlation analysis revealed a significant positive correlation ($p < 0.05$) between the FRP and $ABTS^{\bullet+}$ ($r = 0.98$) and $DPPH^{\bullet}$ ($r = 0.95$) scavenging activities, indicating that aqueous extracts of investigated samples that showed good ferric reducing power also possessed a good ability to scavenge free radicals.

However, the radical scavenging assays are also not consistent with the results obtained for individual and total phenolics analyzed in this study. The activity of complex extracts according to $DPPH^{\bullet}$ is different and closely dependent on the nature of the phenolic compounds present in the extract, because $DPPH^{\bullet}$ is known as a stable and lipophilic radical [47]. Good $DPPH^{\bullet}$ scavenging activity has been previously reported for methanolic and aqueous extracts of field-grown leaves in various blackberry cultivars [38] and methanolic extracts of 'Čačanska Bestrna' leaves also collected in Serbia (83.77%) [5]. Furthermore, several studies have shown good $DPPH^{\bullet}$ scavenging activity for extracts of field-grown leaves of various blueberry cultivars, including leaves of 'Toro' [6,11,12]. Callus cultures of both berry cultivars had significantly lower $DPPH^{\bullet}$ scavenging activity, that is, 3.89% ('Toro') and 4.01% ('Čačanska Bestrna').

Based on the obtained results of the three antioxidant assays, aqueous extracts of field-grown leaves of blackberry 'Čačanska Bestrna' showed the best antioxidant properties, while aqueous callus culture extracts of both berry cultivars had the lowest phenolic content and significantly lower antioxidant properties than the corresponding leaves.

4. Conclusions

This study demonstrated that the aqueous extracts of field-grown and in vitro leaves of blueberry 'Toro' are a better source of total phenolics and flavonoids than those of blackberry 'Čačanska Bestrna', with the former having ten to almost thirty times higher content of PCs as determined by the UHPLC-DAD MS/MS technique. Low TPC and TFC values for both callus cultures were obtained. A total of 20 phenolic compounds were found in all analyzed samples. Quercetin derivatives, chlorogenic acid and gallocatechin were the dominant PCs for both field-grown and in vitro blueberry leaves, whereas quercetin derivatives, syringic and chlorogenic acid were the most abundant phenolic compounds in blackberry. Significant amounts of flavonols such as quercetin-3-*O*-rhamnoside, aesculetin and the aglycones of quercetin and kaempferol were identified only in the in vitro blueberry leaf extract, whereas the phenolic compound profiles of field-grown and in vitro leaves of blackberry were very similar. On the other hand, callus cultures of both berry cultivars had a significantly different PC profile compared to the corresponding leaves.

This study, for the first time, reported the antioxidant properties of in vitro leaves and calluses of these two berry cultivars by the FRP, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities. Opposite to the results of the PC analysis, field-grown and in vitro leaves of blackberry 'Čačanska Bestrna' had better FRP activity, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities than

field-grown and in vitro leaves of blueberry 'Toro', except for ABTS• scavenging activity of in vitro leaves, which did not differ between cultivars. Callus cultures of both berry cultivars showed significantly lower antioxidant activities than the corresponding leaves.

In summary, the aqueous extracts of field-grown and in vitro leaves of blackberry 'Čačanska Bestrna' and blueberry 'Toro' can be a good source of phenolic compounds and exhibit good antioxidant properties, whereas callus cultures of both cultivars can have potential for the production of specific phenolic compounds.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7110420/s1, Table S1: Composition of Murashige and Skoog (MS) basal medium; Table S2: The list of quantified phenolic compounds, with retention times (min), SRM transitions and specified collision energies; Figure S1: Axillary shoot proliferation of blackberry 'Čačanska Bestrna'; Figure S2. Axillary shoot proliferation of blueberry 'Toro'.

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Abbreviations

BA—N6-benzyladenine; 2,4-D—2,4-dichlorophenoxyacetic acid; GA_3—gibberellic acid; IAA—Indole-3-acetic acid; IBA—indole-3-butyric acid; MS—Murashige and Skoog (1962) medium; NAA—α-Naphthaleneacetic acid; PCs—phenolic compunds; TPC—total phenolic content; TFC—total flavonoid content; FRP—ferric reducing power.

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Determination of the Elements Composition in *Sempervivum tectorum* L. from Bulgaria

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Abstract: *Sempervivum tectorum* L. is an evergreen plant with fleshy blue-green leaves forming a rosette. The plant is well-known in alternative medicine and has been used for thousands of years. Traditionally for medicinal purposes, the plant is used as a juice obtained by simple squeezing of fresh plants leaves. The total content of Ca, K, Na, Mg, Mn, Fe, Zn, Cu, Co, Al, V, Cr, Ni, Mo, Ba, Pb, Cd, Hg, As, and Tl in plant leaves of *Sempervivum tectorum* L. sampled from different habitats in Bulgaria was determined after microwave digestion and measurements by inductively coupled plasma mass spectrometry and flame atomic absorption spectrometry. Furthermore, the bioavailable fraction of essential elements Ca, Mg, Fe, Mn, and Zn was defined after extraction with a hydrochloric acid solution, mimicking stomach juice. The total element content showed a high bioavailability of essential human health elements, such as Ca, Mg, Fe, and Zn. Additionally, essential and toxic elements concentrations were quantified in a fresh juice, obtained by squeezing from plant leaves, as most frequently used in folk medicine. The results obtained demonstrated high concentrations of K, Mg, Ca, Zn, and Cu, which could be accepted as an explanation and a further confirmation of the anti-inflammatory action of this plant.

Keywords: *Sempervivum tectorum* L.; mineral content; bioavailable fraction; heavy metals

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1. Introduction

There are over 3000 plant species in Bulgaria, of which more than 600 are used for medicinal purposes. Bulgarian herbs contain a high percentage of biologically active substances [1]. They are rich in various chemical compounds: alkaloids, glycosides, saponins, polysaccharides, tannins, flavonoids, coumarins, essential oils, vitamins, and trace elements. The pharmacological and medicinal action and application of Bulgarian herbs has been an important topic and subject of many studies. One of the most outstanding achievements of the Bulgarian pharmaceutical industry was the creation of the medicinal preparation "Nivalin" by Prof. D. Paskov. The active substance of which is the alkaloid galantamine, extracted from *Leucojum aestivum* L. [2]. Most of the achievements of contemporary medical science are based on bioactive compounds extracted from medicinal plants [3].

Sempervivum tectorum L. (synonym: Sempervivum tectorum var. arvernense (Lecoq & Lamotte) Zonn., Sempervivum tectorum var. andreanum (Wale) O.Bolòs & Vigo) belongs to a large family of Crassulaceae with crassulacean acid metabolism, native to the mountains of southern Europe and cultivated in the whole of Europe.

Sempervivum tectorum L. (houseleek) is an evergreen plant with fleshy blue-green leaves forming a rosette, which grows on dry to fresh sandy soils and in sunny to semi-sunny places. The plant is well-known in folk medicine and has been used for thousands of years.

In recent years, researchers have focused on studies of the characterization of the bioactive ingredients of this plant and their ability to restore liver function [4], their antioxidant properties [5], the potential for wound healing [6], anti-inflammatory action, and analgesic and detoxicating properties [7–9]. Most of these properties of *Sempervivum tectorum* L. are connected with phenolic compounds identified in fresh juices squeezed from plant leaves [10,11]. However, according to the author's knowledge, despite of the numerous uses of *Sempervivum tectorum* L. in folk medicine, it remains poorly known from the viewpoint of systematic investigations into trace element content, element bioavailability, and correlation between essential element content and antioxidant activity.

The trace element content is an essential characteristic of any plant. However, there are no such data for *Sempervivum tectorum* L., neither for environmental safety, nor the effect on human health. The objective of this study was to investigate the level of the elements Ca, K, Na, Mg, Mn, Fe, Zn, Cu, Co, Al, V, Cr, Ni, Mo, Ba. Pb, Cd, Hg, and As as a total content in plant samples of *Sempervivum tectorum* L. obtained from different sampling sites; natural and affected at different levels by human activities. The urban soil (A) is from an urban park close to center of the city, and the fertilized soil (C) is from land used for agriculture purposes for many years. The rural soil (B) and mountain soil (D) might be accepted as natural; however, with varying composition. The bioavailable fraction of essential elements Ca, Mg, Fe, Mn, and Zn, defined according to a standard procedure for element migration in hydrochloric acid that mimics food digestion processes in the stomach, was quantified. Additionally, K, Ca, Mg, Na, Fe, Mn, Zn, Al, Cu, and Cr were determined in the freshly squeezed juice from plant leaves, as directly used in folk medicine against ear pain.

2. Materials and Methods

Plant Material. *S. tectorum* plants were from different habitats, grown on city soils (A), village soils (B), fertilized soils (C), and mountain soils (D). The leaves of the plants were removed, thoroughly washed with deionized water to remove all possible external contaminants, and used immediately for:

(i). the preparation of fresh juice after squeezing;
(ii). the preparation of a fresh homogeneous sample mix after milling for the determination of bioavailable fraction;
(iii). the preparation of dry mass after oven drying to a constant weight at 40 °C and homogenization by careful grinding.

Sample preparation before analysis:

Reagents: 67% HNO_3 (supra pure, Merck, Darmstadt Germany); 30% H_2O_2 (supra pure, Merck, Darmstadt Germany); 37% HCl (p.a. Sigma-Aldrich, Darmstadt Germany).

2.1. Determination of Total Content of Elements

A dry sample of around 0.5 g was weighed in Teflon vessels of a microwave digestion system, 6 mL 67% HNO_3 and 2 mL 30% H_2O_2 were added, and samples were left overnight. Microwave digestion was performed for 20 min: 10 min to reach 180 °C and 10 min maintained at this temperature. After cooling, samples were transferred to a 50 mL volumetric flask and diluted up to the mark with deionized water. A blank sample was passed through the whole analytical procedure.

2.2. Determination of Bioavailable Fraction in Fresh Leaves

A sample of 2.0 g of fresh leaves was milled with 50 mL deionized water in a plastic container. After that, 50 mL 0.14 mol L^{-1} HCl were added, and the mixture was shaken for at least 1 min. The suspension was left for several minutes to settle, and the pH of the clear supernatant was measured. If the pH was above 1.5, 2 mol L^{-1} HCl solution

was added drop-wise while mixing until the pH reached values between 1.0 and 1.5. The container was closed and agitated at 37 ± 2 °C for 1 h. After that, the suspension was left for a further 1 h at 37 ± 2 °C. The mixture was protected from daylight. The solid matter was separated by centrifugation and, if necessary, filtrated through a membrane filter (0.22 μm) to remove all solid particles. The separation should be completed as soon as possible after completing the standing time; centrifuging should take no longer than 10 min. Next, the obtained solution was evaporated on a hot plate to 2–3 mL, 3 mL of conc. HNO_3 was added for digestion of the organic components, and, finally, the sample was quantitatively transferred to a 25 mL flask and made up with deionized water [12].

2.3. Determination of Elements in Juice Obtained by Squeezing of Fresh Leaves

A sample of 2.0 g juice (obtained after filtration of fresh juice through a 0.22 μm membrane filter) was transferred in a glass beaker and treated with 1 mL 67% HNO_3 on a hot plate. After 1 h, the solution was cooled and diluted in a 10 mL volumetric flask with distilled water.

Apparatus for quantitative measurement of chemical elements:

Flame atomic absorption spectrometry: The content of Fe, K, Mn, Mg, Na, and Zn was measured by flame atomic absorption spectrometry (Thermo Electron—SOLAAR Mkll M5 series, UK) in an air/acetylene flame under optimized instrumental parameters. The content of Ca was measured in N_2O/acetylene flame, using the same instrument. Stock standard solutions of Ca, Fe, K, Mn, Mg, Na, and Zn (1.000 g L^{-1}(Merck)) were used for the preparation of diluted working standards.

Inductively coupled plasma mass spectrometry: The content of As, Al, Ba, Cd, Co, Cr, Cu, Hg, Mo, Ni, Pb, and V was measured by ICP-MS using an inductively coupled plasma mass spectrometer "X SERIES 2"—Thermo Scientific, USA with a 3 channel peristaltic pump; concentric nebulizer; Peltier-cooled spray chamber (4 °C); Xt interface option; Ni cones. Optimized instrumental parameters: forward plasma power of 1400 W; plasma gas flow 13 L min/L; nebulizer flow 0.85 L/min; dwell time 30 ms; measurements 3 × 30 scans. Stock standard solutions: multielement standard solution 5 for ICP (TraceCERT®, Merck), 1000 mg/L As (Fluka, Sigma-Aldrich) and 1000 mg/L Hg (Fluka, Sigma-Aldrich) were used for the preparation of diluted working standard solutions for calibration of ICP-MS.

The accuracy of the analytical procedure used was validated by the analysis of certified reference material NIST SRM 1573a Tomato leaves. The very good agreement with the certified values and the recoveries above 95% achieved for all certified elements confirmed the reliability of the results obtained for total element contents (see Table 1). Limit of detection and limit of quantification was calculated for each element based on standard deviation of blanks sample for the respective procedures using 3 σ criterium (LOD) and 10 σ criterium (LOQ). Calculated values for LOD and LOQ are presented in Table 1.

2.4. Statistical Analysis

Data for the concentrations of chemical elements were processed to obtain the mean and standard deviation of the mean (SD). One-way analysis of variance, followed by a Student's *t*-test was used to compare the mean values. A value of $p < 0.05$ was considered to be statistically significant.

Table 1. Results and recoveries for chemical element contents (mg/kg) determined in NIST SRM 1573a Tomato leaves (three parallel determinations).

Element, mg/kg	Determined (Mean ± sd)	Certified (Mean ± sd)	Recovery, % (Mean)	LOQ/LOD, mg/kg
Al (ICP-MS)	594 ± 4	598.4 ± 7.1	99.3	0.10/0.35
As (ICP-MS)	0.1088 ± 0.056	0.1126 ± 0.0024	96.6	0.02/0.06
Cd (ICP-MS)	1.456 ± 0.016	1.517 ± 0.027	96	0.02/0.05
Ca (FAAS)	49,441 ± 342	50,450 ± 550	98	2/6
Cr (ICP-MS)	1.92 ± 0.04	1.988 ± 0.034	96.6	0.05/0.15
Co (ICP-MS)	0.5588 ± 0.021	0.5773 ± 0.0071	96.8	0.02/0.06
Cu (ICP-MS)	4.56 ± 0.11	4.70 ± 0.14	97	0.1/0.3
Fe (FAAS)	363.8 ± 2.1	367.5 ± 4.3	99	3/10
Mn (FAAS)	243.8 ± 9.3	246.3 ± 7.1	99	3/10
Hg (ICP-MS)	0.0329 ± 0.0043	0.0341 ± 0.0015	96.5	0.02/0.06
Ni (ICP-MS)	1.536 ± 0.031	1.582 ± 0.041	97.1	0.02/0.05
K (FAAS)	26,490 ± 312	26,760 ± 480	99	5/15
Na (FAAS)	134.3 ± 2.5	136.1 ± 3.7	98,7	5/15
V (ICP-MS)	0.809 ± 0.042	0.835 ± 0.034	96.9	0.02/0.06
Zn (FAAS)	30.02 ± 0.56	30.94 ± 0.55	97	1/3

3. Results

The profile of chemical elements in plants depends on the geochemical characteristics of the soil [13] and on the ability of plants to selectively accumulate minerals essential for their growth. For given plants, the content of mineral and trace elements is characteristic and will be affected by different factors, such as the physical and chemical properties of the soil, application of natural and artificial fertilizers, and climatic conditions of the region. The results obtained for the total content of elements in *Sempervivum tectorum* L. are presented in Tables 2–4.

Table 2. Essential (basic) element contents in dry samples.

	K g kg^{-1}	Ca g kg^{-1}	Mg g kg^{-1}	Na mg kg^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}	Zn mg kg^{-1}
city soils (A), number of plant samples-4							
mean	18.0	107	10.6	93.6	325	35.4	49.2
min	9.36	102	4.39	16.4	188	13.9	45.1
max	29.9	115	12.3	234.9	398	65.4	51.7
village soils (B), number of plant samples-5							
mean	11.1	116	11.6	206	384	30.7	79.0
min	7.59	84.7	7.71	176	328	17.4	42.2
max	12.9	132	18.2	230	491	50.9	135
fertilized soils (C), number of plant samples-4							
mean	26.3	66.2	5.97	74.4	358	273	30.5
min	10.7	60.7	3.4	67.2	243	102	26.7
max	31.4	103	7.81	112	427	283	44.8
mountain soils (D), number of plant samples-4							
mean	15.7	61.0	5.68	125	247	12.1	29.2
min	7.21	57.3	3.84	102	197	10.5	25.5
max	21.3	85.1	10.5	131	343	18.4	37.8

Table 3. The content (mg/kg) of non-essential elements in dry samples.

	Al mg kg^{-1}	Co mg kg^{-1}	Cu mg kg^{-1}	Ba mg kg^{-1}	Mo mg kg^{-1}	V mg kg^{-1}	Cr mg kg^{-1}
A							
mean	23.3	0.46	7.91	51.0	0.86	<0.02	0.42
min	17.0	0.32	5.63	48.5	<0.02 *	<0.02	0.37
max	32.6	0.56	11.0	53.9	2.53	<0.02	0.45
B							
mean	61.1	0.39	8.12	65.4	1.96	0.10	0.63
min	38.5	0.35	5.33	50.6	<0.02	<0.02	0.49
max	99.6	0.47	10.7	74.0	5.62	0.23	0.90
C							
mean	257.6	2.13	9.14	145.8	<0.02	0.05	0.76
min	94.5	1.12	7.43	85.3	<0.02	<0.02	0.37
max	301.2	2.54	12.32	153.2	<0.02	0.17	0.94
D							
mean	18.5	0.24	7.32	38.7	<0.02	<0.02	0.38
min	13.4	0.05	4.91	29.5	<0.02	<0.02	0.23
max	21.3	0.32	8.94	50.4	<0.02	<0.02	0.42

* Limit of detection.

Table 4. The total content (mg/kg) of toxic elements (Cd, Pb, As, Hg, and Ni) in dry samples.

	Cd mg kg^{-1}	Pb mg kg^{-1}	As mg kg^{-1}	Hg mg kg^{-1}	Ni mg kg^{-1}
A					
mean	0.27	2.66	0.14	0.05	2.03
min	0.17	1.56	<0.02	<0.02	1.32
max	0.46	3.99	0.36	0.10	2.38
B					
mean	0.23	1.05	0.05	0.03	2.40
min	<0.02	0.63	0.03	<0.02	2.19
max	0.26	1.42	0.09	0.05	2.73
C					
mean	0.10	3.18	0.07	0.03	4.51
min	<0.02	1.43	<0.02	<0.02	1.29
max	0.27	4.02	0.12	0.05	4.78
D					
mean	<0.02	1.29	0.08	0.02	0.89
min	<0.02	0.54	<0.02	<0.02	0.32
max	<0.02	1.78	0.11	0.05	1.15

The bioavailable content of Ca, Mg, Zn, Mn, and Fe in fresh leaves of *Sempervivum tectorum* L. is depicted in Table 5.

The concentrations of elements in juice obtained from fresh leaves are presented in Table 6.

Table 5. Bioavailable content of Ca, Mg, Zn, Mn, and Fe in fresh leaves of *Sempervivum tectorum* L. as a mean values (RSD for all samples varied between 3–8%).

	Ca g kg^{-1}	Mg g kg^{-1}	Zn mg kg^{-1}	Mn mg kg^{-1}	Fe mg kg^{-1}
B	5.07	0.40	3.55	1.97	16.5
C	2.95	0.44	3.58	9.02	20.0
D	3.16	0.37	2.37	1.71	12.3

Table 6. Element concentrations (mg/L) in fresh juice from *Sempervivum tectorum* L.

Elements	A	Elements	A
K, mg L^{-1}	133	Na, mg L^{-1}	0.7
Ca, mg L^{-1}	561	Zn, mg L^{-1}	1.95
Mg, mg L^{-1}	2845	Al, mg L^{-1}	3.45
Fe, mg L^{-1}	0.07	Cu, mg L^{-1}	0.28
Mn, mg L^{-1}	2.40	Cr, mg L^{-1}	0.29

4. Discussion

As seen from Table 1, *Sempervivum tectorum* L. contains an extremely high calcium content, exceeding by between 3–10 times the concentrations of the second highest content element, K. No statistically significant differences were found for the total content of essential elements (except Ca and Mn) in plants from different regions, confirming the bio-uptake ability of plant toward essential elements [14]. Unexpectedly a higher total content of Ca was observed in rural and urban plants in comparison with plants from fertilized and mountain regions. Significantly higher concentrations of Mn were determined in the plants grown on fertilized soils, which might be explained by the high bioavailable Mn content in these soils, as the same concentrations were measured in other herbs from the same region. The total content of essential elements presented at lower concentration levels in *Sempervivum tectorum* L. is close to the content of these elements in other herbs from these regions [15,16].

Table 2 lists the results obtained for some nonessential elements. As can be seen, the plants grown on agricultural (fertilized) soils differed from the others with their higher concentrations of Al, Co, and Ba. However, only the concentrations of cobalt were surprising, as Al of such and higher concentrations is found in herbs from this region [15].

Another critical aspect is the good quality control of medicinal herbs, to protect consumers from contamination, as many medicinal herbs and their mixtures can present a health risk due to toxic elements [17].

Toxic element levels in raw plant material or prepared products/extracts/infusions is regulated by documents at global, national, or regional level. Strict control of contaminant levels and their minimization is required by the World Health Organization (WHO) through guidelines such as the good agricultural and collection practices (GACP) for medicinal plants and good manufacturing practices (GMP) for herbal medicines. Maximal values for toxic elements in herbal drugs and extracts have been discussed and compared by several authors [18–20].

According to the World Health Organization, cadmium concentrations and lead in herbal medicines and products are regulated at 0.3 mg kg^{-1} Cd and 10.0 mg kg^{-1} Pb [18]. In different countries, the law sets lower limits, and a very good comparison of the various permissible limits is presented by Luo et al. [20]. As shown in Table 3, the concentrations of toxic elements meet the requirements of the WHO, and only in one single case was the cadmium concentration exceeded, for urban soil. Expectedly, the results for elements such as As, Cd, and Pb are highest in plants grown on urban soils. Most probably, in this case both soil pollution and aerosol deposition are responsible for the high toxic element content. Although, it is clearly important to harvest medicinal plants from clean sites

without anthropogenic influences such as mountain regions. A relatively high content was determined for Ni and Pb in plants grown on fertilized soils, most likely connected with Ni and Pb contamination by the phosphate fertilizers applied.

In this study, sampling for all studied plants and sampling sites was performed in the summer season, with some efforts to use plants in the same vegetation period. Taking into account that *Sempervivum tectorum* L. is a perennial plant, additional research is required to elucidate any correlation between plant age and chemical element content.

In Table 4, the results found for the operationally defined bioavailable content (see Section 2.3) of Ca, Mg, Zn, Mn, and Fe in fresh leaves of *Sempervivum tectorum* L. (after two hours of treatment in pH 1.0–1.5) are depicted. Plants growing on mountains, villages, and fertilized soils were used. The percentage content of bioavailable fraction varied between 4–14% for all studied essential elements (Table 7). It should be pointed out that the content of Ca and Fe, which might be accepted as being most responsible for the health functions of *Sempervivum tectorum* L., is almost constant in the bioavailable fractions from all samples. High concentrations of Ca in this fraction justify the use of *Sempervivum tectorum* L. as a national remedy for the treatment of gastric ulcers, possibly because of the beneficial calcification effect.

Table 7. Bioavailable concentrations of Ca, Mg, Zn, Mn, and Fe as a percentage of total content.

	Ca Bioavaible Fraction, %	Mg Bioavaible Fraction, %	Zn Bioavaible Fraction, %	Mn Bioavaible Fraction, %	Fe Bioavaible Fraction, %
B	4.37	3.45	4.49	6.42	4.30
C	4.46	5.63	11.7	3.30	5.59
D	5.18	6.51	8.12	14.1	4.98

Although the concentration of Mn is still highest as a bioavailable concentration, the degree of extraction was significantly lower, most probably depending on the different Mn species present in the leaves. Therefore, it might be assumed that the Mn bio-uptake would be highest from agricultural (fertilized) soils, most probably because of suitable pH values.

Determination of elements in juice from fresh leaves. Fresh juice obtained by squeezing leaves from *Sempervivum tectorum* L. was widely used as folk medicine against ear pain. As shown in Table 6, this effect can most probably be explained by the high Mg concentrations, analogous to the pharmaceuticals used for external application (Mg-gels or Mg-oils) with anti-inflammatory and regenerative actions and improved blood circulation [21–23]

5. Conclusions

The total element contents and the bioavailable fraction of essential elements Ca, Mg, Zn, Mn, and Fe were determined in leaves of *Sempervivum tectorum* L. The control of the quality of medicinal plants used in traditional medicine and pharmacy is an important step for consumer protection from contamination and health risks. The determination of toxic element content in plants grown on different soils clearly shows the contamination of plants from urban soils and plants fertilized with phosphate fertilizers. The high bioavailable concentrations of essential elements could explain the wide use of this plant in folk medicine. For example, the high Mg content in fresh juice is responsible for its anti-inflammatory action and application as an ear pain reliever.

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Review

Recent Advancements in Enhancing Antimicrobial Activity of Plant-Derived Polyphenols by Biochemical Means

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Abstract: Plants are a reservoir of phytochemicals, which are known to possess several beneficial health properties. Along with all the secondary metabolites, polyphenols have emerged as potential replacements for synthetic additives due to their lower toxicity and fewer side effects. However, controlling microbial growth using these preservatives requires very high doses of plant-derived compounds, which limits their use to only specific conditions. Their use at high concentrations leads to unavoidable changes in the organoleptic properties of foods. Therefore, the biochemical modification of natural preservatives can be a promising alternative to enhance the antimicrobial efficacy of plant-derived compounds/polyphenols. Amongst these modifications, low concentration of ascorbic acid (AA)–Cu (II), degradation products of ascorbic acid (DPAA), Maillard reaction products (MRPs), laccase–mediator (Lac–Med) and horse radish peroxidase (HRP)–H_2O_2 systems standout. This review reveals the importance of plant polyphenols, their role as antimicrobial agents, the mechanism of the biochemical methods and the ways these methods may be used in enhancing the antimicrobial potency of the plant polyphenols. Ultimately, this study may act as a base for the development of potent antimicrobial agents that may find their use in food applications.

Keywords: polyphenols; antimicrobial activity enhancement; ascorbic acid; Maillard reaction products; laccase–mediator system; horseradish peroxidase–H_2O_2 system

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1. Introduction

The quality and safety of food products are compromised by the loss of nutrients, sensory attributes, and microbial growth. The prior can be in terms of off odor, off flavor, discoloration, or production of toxic compounds as metabolic end products of the saccharolytic, proteolytic, pectinolytic, and lipolytic enzymes, which ultimately leads to food poisoning or intoxication. Ensuring food safety and meeting the demand for food without synthetic chemical preservatives has led to increased interest in natural alternatives to inactivate microorganisms and enzymes in food [1,2]. Although attempts have been made to produce additive-free foods, it is unlikely that the current marketing system could exist without the use of antimicrobials. In addition, requirements for toxicology safety have limited the ability of the industry to develop new chemical antimicrobials [3]. Therefore, it is essential for the food industry to find new and natural antimicrobial food alternatives.

Phytochemicals can be recovered from plant products and used as ingredients in food and cosmetics, as healthy antimicrobials, and as alternatives to chemical preservatives. A typical feature of plants is their ability to synthesize a wide range of phyto-compounds (i.e., secondary metabolites), which play essential roles in the interaction of the plant with its environment [4]. They can be structurally divided into five major groups: phenylpropanoids, flavonoids, polyketides, terpenoids, and alkaloids. Furthermore, it is increasingly clear that several phytocompounds in fruits and vegetables of different chemical classes are beneficial to human health [5,6]. Interestingly, several phytochemicals such as simple phenolic acids,

polyphenols, terpenes, isothiocyanates, polyacetylenes, etc., also exhibit antimicrobial properties. There is sufficient evidence supporting the potential of plant-derived phytochemicals as natural antimicrobial agents [7–9].

However, only a few natural antimicrobials have found practical application in the food industry. Their use in foods as preservatives is often limited due to the need for high concentrations to achieve the desired activity, which may modify the sensory characteristics of food by making it unacceptable [10]. Another limitation is the interaction of natural antimicrobial with complex food matrices, mainly with hydrophobic compounds such as lipids [11]. Nonetheless, polyphenols can interact with proteins through hydrophobic or hydrophilic interactions, leading to the formation of soluble or insoluble complexes [12].

The "antimicrobial potency" of polyphenols can be altered and enhanced by biochemical means that could allow their application as antimicrobial agents. This means that the antimicrobial effect can be improved by reducing the effective concentration of the plant-derived compounds [13]. Biochemical and physiological studies have provided a large body of evidence to surmise that plant-derived polyphenols can be well adapted to achieve promising and potent antimicrobials for use in foods, ensuring microbial safety of foods without chemical additives [14,15]. Such biochemically modified natural ingredients would positively affect food preservation without compromising the sensory attributes and health of the consumers.

2. Plant Polyphenols as Antimicrobials

Recent studies have shown that plant compounds used as natural antimicrobials are safe alternatives to chemical additives [16,17]. Natural antimicrobials' mechanism of action includes cell membrane rupture, defective nucleic acid mechanisms, decay of the proton motive force, and depletion of adenosine triphosphate (ATP). The antimicrobials from plants (polyphenols, essential oils) use the aforementioned mechanisms of action against foodborne bacteria [18]. Amongst all secondary metabolites in plants, polyphenols are the ones that play multiple essential roles in plant physiology, also in addition having potential health-benefiting properties such as having antioxidant, antiallergic, anti-inflammatory, anticancer, antihypertensive, and antimicrobial features [19,20]. Basically, they are divided into flavonoids and non-flavonoids, on the basis of their chemical structure.

2.1. Flavonoids

Flavonoids, such as catechins, flavones, and flavonols, have antifungal, antiviral, and antibacterial activities [21]. The antimicrobial activity of the flavonoid quercetin is attributed to the inhibition of the enzyme DNA gyrase and (-)-epigallocatechin gallate, which was reported to inhibit the energy metabolism [21]. Flavonoids, especially catechins and proanthocyanidins (due to antioxidant properties), have been proposed to neutralize bacterial toxic factors originating from *Vibrio cholerae*, *Staphylococcus aureus*, *Vibrio vulnificus*, *Bacillus anthracis*, *Clostridium botulinum* [22]. The citrus flavonoids, such as apigenin, kaempferol, quercetin, and naringenin, are effective antagonists of cell–cell signaling [23]. Recent reviews have provided lines of evidences on the antimicrobial activity of plant flavonoids along with their mechanism of actions [24,25].

2.2. Non-Flavonoids

Phenolic acids (benzoic, phenylacetic, and phenylpropionic acids) have been found to inhibit pathogenic and non-pathogenic bacteria and fungi such as *Escherichia coli*, *Lactobacillus* spp., *S. aureus*, *Pseudomonas aeruginosa* and *Candida albicans* [26]. Hydroxycinnamic acids (caffeic, coumaric, ferulic, and sinapic acids) have been found to inhibit *Bacillus cereus* and *S. aureus*; *P. fluorescens* [27]. In addition, the antibacterial activity of caffeic, ferulic, and p-coumaric acids against *E. coli*, *S. aureus*, and *B. cereus*, with p-coumaric acid being effective against *E. coli*, has been reported [27]. Hydroxycinnamic acids (i.e., nitrobenzoate, p-aminobenzoate, ethyl aminobenzoate, ethyl- and methyl-benzoate, salicylic acid, trans-cinnamic acid, trans-cinnamaldehyde, ferulic acid, o-acetoxy benzoic acid, and anthranilic

acid) have been found to inhibit aflatoxins production from *Aspergillus flavus* and *Aspergillus parasiticus* [28]. Additionally, furocoumarins present in carrots, celery, citrus fruits, parsley, and parsnips have been reported for their antimicrobial activity against *E. coli* O157:H7, *Erwinia carotovora*, *Listeria monocytogenes*, and *Micrococcus luteus* [29].

The antibacterial properties of some common foods and beverages such as coffee against *Legionella pneumophila* and *E. coli* O157:H7 are attributed to its compounds such as caffeic acid, chlorogenic acid, and protocatechuic acid [30,31]. Furthermore, tea (*Camellia sinensis*) has also been found to display antimicrobial properties [32–34] through its predominant catechin, epigallocatechin gallate, against methicillin-resistant *S. aureus* (MRSA). The compound E-cinnamaldehyde has been found to significantly contribute to the antimicrobial properties of cinnamon stick extract (Ext) against *B. cereus*, *E. coli*, *L. monocytogenes*, *S. aureus*, and *Salmonella* [35].

2.3. Extraction of Polyphenols from Plant Products

Extraction methods have been developed recently using modern technology. These methods use fewer or no organic solvents, thereby minimizing environmental and health impacts and maximizing the yield of desired polyphenols by selective extraction [36]. Advanced methods such as microwave-assisted, ultrasound-assisted, pulsed -electric-field-assisted and enzyme-assisted extractions, as well as pressurized liquid and supercritical fluid extractions, are given prime importance these days to extract desired polyphenols from the plant products [37,38]. One of the recent studies has suggested extraction of non-extractable or bound polyphenols by pretreatment using the aforementioned methods, which are further cleaved using acid, alkaline, or enzyme treatments, followed by purification step using solid-phase extraction column chromatography and finally storage step using lyophilization [39]. Studies have illustrated that the bioavailability and yield of polyphenols are one of the most important factors of their antimicrobial activity [40,41]. However, along with these factors, their structure has also been found to play a critical role in their antimicrobial activity [42,43]. The relationship between the structure of polyphenols and their antimicrobial activity is elaborately illustrated in the proceeding section.

3. Antimicrobial Activity and Structural Relationship of Plant-Derived Polyphenols

The structural diversity of polyphenols is immense, and the impact of antimicrobial action they produce against microorganisms depends on their structural configuration [44]. For instance, Phenolic acids inhibit the activity of bacterial enzymes, disrupting their metabolism and depriving the substrates necessary for growth. The hydroxycinnamic acids (p-coumaric acid, caffeic, and ferulic acid) induced higher ion leakage and a more significant influx of protons into the cells, compared with hydroxybenzoic acids, gallic, vanillic, and syringic acid [45]. Additionally, these hydroxycinnamic acids have been found to meet Lipinski's rules, proving their functional potential as drugs and antimicrobial agents. The relationship between chemical structure and biological activity has received considerable attention in recent years because it allows the prediction of chemical toxicity or bioactivity without an inordinate amount of time and effort.

The potency of an antimicrobial is attributed to its structural characteristics. The relationship of the antimicrobial activity of plant polyphenols is classified into four types: (1) position of functional groups (FNG), (2) number of FNG, (3) presence of C2=C3 double bond, and (4) type of FNG.

3.1. Position of Functional Group

The structural antimicrobial activity of the major plant polyphenols, i.e., flavonoids, is well documented [46]. The amphipathic features of flavonoids play an essential role as far as antibacterial properties are concerned [47]. The hydrophobic substituents such as prenyl groups, alkylamino chains, alkyl chains, and nitrogen or oxygen-containing heterocyclic moieties usually enhance the antibacterial activity of all flavonoids [48]. Different classes of

flavonoids, mainly chalcones, flavanes, and flavan-3-ol exhibits better antimicrobial activity due to variation in the position of the functional group attached to the rings [46].

3.1.1. Chalcones

Several studies have suggested that chalcones with a lipophilic group such as isoprenoid and methoxy groups at positions 3′, 5′, and 2′ of ring A are the most potent inhibitors of MRSA strains [49]. Based on the activity of isobavachalcone (MIC: 30 μg/mL), (Figure 1A), the authors of [50] suggested the that A ring with a prenyl group displays adequate antimicrobial activity, but cyclization or addition of the prenyl group to B ring in addition to the A ring decreases this activity. Likewise, the hydroxy group at 4′, 4, and 6 of A and B rings increase the antimicrobial activity. For example, kuraridin and 7,9,2′,4′-tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC) compounds with the same structure, with only one difference in the OH of the B ring (2 and 4 instead of 4 and 6), showed high activity against the MRSA strain [51].

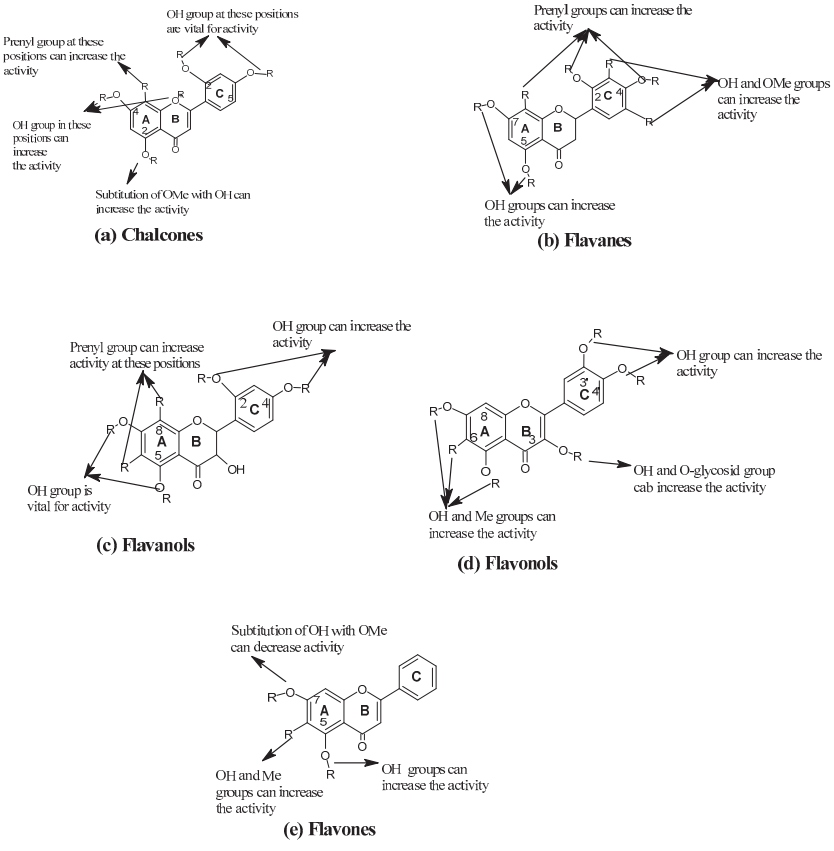

Figure 1. Structure–activity (SAR) relationship of important flavonoids: (**a**) in chalcones, substitution of OMe, OH, and prenyl group in ring A, OH group in ring B, and OH groups in 2′ and 4′ position of ring C enhances antimicrobial activity; (**b**) on flavanes, substitution of prenyl and OH groups at 5′and 7′ positions in ring A and OH, OMe groups at 3′ and 5′ position and prenyl at 4′ position in ring C enhances antimicrobial activity; (**c**) in flavanols, substituting OH group at 5′ position and prenyl at 8′ position of ring A, and OH groups at 2′, 4′ position can enhance antimicrobial activity; (**d**) in flavonols, replacing OH and Me group in ring A, OH, O-glycoside group in ring B and OH groups in ring C can improve antimicrobial activity; (**e**) In flavones substitution of OH group at 5′, OH, Me groups at 6′, and OH, OMe group at 7′ of ring A can improve antimicrobial activity.

3.1.2. Flavanes and Flavanols

Flavanes with a prenyl group at the A ring have been found to be the most potent antibacterial compounds against *S. aureus*. It is established that the number and position of prenyl groups on this ring increase antimicrobial activity [52]. The presence of the hydroxy groups at different positions on A and B rings has also been reported to improve antibacterial activity (Figure 1B,C). The compound 3′-O-methydiplacol with OH at the 5, 3′, and 4′ positions of the A and B rings, respectively, as well as the geranyl group at C-6 and OMe at C-5′, showed satisfactory (i.e., MIC value of 4 μg/mL) activity against *S. aureus* [53]. additionally, sophoraflavanone with isogeranyl at C-8 and OH at 3, 2′, and 4′ at A and B rings were active (i.e., MIC value of 7.3 μg/mL) against *S. aureus* [51]. The position of prenyl, hydroxyl, and especially methoxy groups at positions 5 and 7 of the A ring, increased the antibacterial effect of flavanes and flavones [54]. Furthermore, different substitutions on position 3 of the C ring with a hydroxyl or an *O*-glycoside group could enhance the antimicrobial activity of certain flavones [46]. One of the earlier findings also suggested that the tetraflavonoids without OH on the C ring showed moderate activity against *E. coli* [55].

3.1.3. Flavonols

In the A ring, many studies have confirmed that hydroxylation at positions 5 and 7 together are critical for the antibacterial activity of flavonols against *S. aureus* strains [56]. In addition, hydroxylation on the B and C rings also increased the antimicrobial activity of these compounds. A comparison of compounds with the same structure showed that kaempferol with a hydroxy group at C-4′ had less activity than galangin (without OH at C-4′) against *S. aureus* (Figure 1D) [47]. The number of glycosyl groups instead of the hydroxy group at position 3 has been found to have a significant effect on antibacterial activity. For example, among the compounds extracted from *Maytenus buchananii*, quercetin-3-O-[α-L-rhamnopyranosyl-(1 → 6)-β-D glucopyranoside] with a disaccharide group at the same position was the better inhibitor of *S. aureus* growth than amentoflavone-7″,4‴-dimethyl-ether with monosaccharide group (quercetin-3-O-β-D-glucopyranoside) [57]. Substitution of the methoxy group at position 3 decreased the antimicrobial activity. For example, piliostigmol (with OMe and Me groups at positions 6 and 7 of the A ring and OH at position 3) was more active against *S. aureus* than 6-C-methylquercetin-3,3′,7-trimethyl ether (with OMe at the C-3 position) [58].

3.1.4. Flavones

Studies conducted on the antibacterial activity of flavones [59] suggested that at least one hydroxy group in the A ring (especially at C-7) is vital for antibacterial activity. Hydroxyl groups in other positions such as C-5 and C-6 can also increase the antibacterial action [60]. However, the substitution of OH with OMe at C-7 was seen to reduce the antibacterial activity. For instance, the compound 5,7-dihydroxy-flavone with two OH at positions 5 and 7 has been found to be more potent against *Ralstonia solanacearum* (i.e., MIC: 25 and 300 μg/mL) compared to 5-hydroxy-7-methoxy-flavone with OMe at position 7 and OH at position 5 (Figure 1E) [61]. The importance of the –OH group at position 5 of flavones for their antimicrobial activity against MRSA strains has also been reported [62]. One of the investigations on plant isoflavonones suggested that the hydroxyl group's C-5, 6 and 7 position is crucial for antimicrobial action [63]. The presence of the prenyl (C5) group at position 6 without cyclization of this substituent with the A ring has also been reported to improve antibacterial activity [52].

3.2. Number of Functional Groups Attached

The number of functional groups attached has been found to have a significant influence on antimicrobial activity. The 2′, 4′- or 2′, 6′-dihydroxylation of the B ring and 5, 7-dihydroxylation of the A ring in the flavanone structure are essential for anti-MRSA activity [64]. Moreover, 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two

or three additional hydroxyl groups at the 7′, 2′, and 4′ positions inhibited the growth of *Streptococcus mutans* and *S. sobrinus* [65]. Caffeic acid had higher antimicrobial activity than p-coumaric acid due to the additional –OH group on the phenolic ring of the former compound [66].

3.3. Presence of C2=C3 Double Bond

It has been observed that flavanones with C2=C3 are more active than the corresponding flavones. For example, naringenin showed antibacterial effects on all the tested bacteria, whereas apigenin showed almost no effect [67]. In addition, the C2=C3 double bond was found to be responsible for the antifungal activity of 5,7-dihydroxyflavonoids, while hydrogenation of the C2=C3 bond reduced the antifungal effect [68]. The flavonoids apigenin luteolin, dinatin, and daidzein, C2=C3 had better anti-influenza virus activities, compared with catechin and epicatechin belonging to the flavanols class of compounds that lack the C2=C3 bond [69].

3.4. Type of Functional Group

The hydrophobic substituents such as prenyl groups, alkylamino chains, alkyl chains, and nitrogen- or oxygen-containing heterocyclic moieties have been reported to enhance the activity of all the flavonoids [48]. Variation in the antimicrobial activity of polyphenols also depends on variation in the functional group they have [70]. The substitution of the phenyl moiety by a propyl or a methyl group has been found to be deleterious for the antibacterial effect against *S. aureus* and *B. subtilis* [71]. This negative antimicrobial effect was also observed against *L. monocytogenes*, when substituting the phenyl with the propyl moiety [71].

The naphthoquinone 5,8-dihydroxy-1,4-naphthoquinone without chlorine was very active against three Gram-positive (*S. aureus*, *B. subtilis*, and *L. monocytogenes*) and three Gram-negative (*E. coli*, *P. aeruginosa*, and *S. Enteritidis*) strains but in a lower extent against *P. aeruginosa*. The compound 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone with chlorine was significantly less active against *E. coli* and *P. aeruginosa* [70]. The presence of gallic or galloyl moieties was found to promote the antibacterial activity of epigallocatechin gallate by inducing damage to the bacterial membrane [72]. The antibacterial action of caffeic acid and their alkyl esters against specific strains of *S. aureus* and *E. coli* showed that longer alkyl side chains were more effective against the Gram-positive bacterium, while caffeic acid esters with medium length alkyl side chain were more effective against the Gram-negative bacterium which was also far less susceptible to caffeic acid and its esters [73].

4. Enhancement of Antimicrobial Activity of Plant Derived Polyphenols by Biochemical Methods

Higher potency of the antimicrobial or their use in low concentration is preferred in food application to avoid any changes in the organoleptic properties and minimize interaction with the complex food matrices [14,74]. The following biochemical means may be used to obtain high potent antimicrobials.

4.1. Enhancement Using Ascorbic Acid and Transition Metals

One of the promising approaches to enhance antimicrobial activity by non-enzymatic means can be mild oxidation of plant phytochemicals, particularly polyphenols by reactive oxygen species (ROS), using ascorbic acid (AA) and transition metals (Cu (II), Fe (II), Fe (III) systems. However, the reaction mechanism of AA oxidation in the presence of transition metals is still unclear, and different mechanisms have been proposed.

In one study, the AA and Cu^{2+} reaction were projected to yield one mole of hydrogen peroxide (H_2O_2) and one mole of dehydro-ascorbic acid in the pH range of 2.6–9.3 [75]. Additionally, it has been assumed that H_2O_2 is formed when AA reacts with Cu(II) in the presence of O_2 within a pH range of 2.5–4.0 [76]. At physiological pH (7.0), H_2O_2 production increased when the copper (Cu^{2+}) concentration was deficient, compared with

AA [77]. It was further postulated that the ascorbate mono-anion would dominate when the pH is over 4.25. This form can further be oxidized with the concomitant reduction of copper II to copper I [78]. At pH higher than 4.25, rapid redox cycling of copper generates superoxide, peroxide, and hydroxyl radicals via a copper assisted Fenton reaction, and at a pH lower than 4.25, the level of superoxide in the solution decreases as superoxide anion reacts with hydrogen to form the hydroperoxyl radical ($HO_2^{\bullet}$) [78].

The reaction product of AA and Cu^{2+} leads to cleavage of viral and plasmid DNA, which could be withdrawn in the presence of metal chelators such as EDTA, stating that copper plays an essential role in the oxidation of AA [79]. The reaction was also withdrawn in the presence of the catalase enzyme, confirming the fact that H_2O_2 is mainly produced in the AA/Cu^{2+} reaction [79]. The hydroxyl radical generated by the AA/Cu^{2+} system is lesser than that generated by the ascorbate/H_2O_2 system [80]. It has also been reported that transition metal ions, such as Fe (III) and Cu (II), are reduced by ascorbate. Their lower oxidation states (e.g., Fe (II) and Cu (I), respectively) (Equations (1) and (2) below) may further give rise to Fenton reactions with H_2O_2, producing hydroxyl radicals [81].

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (2)$$

In the presence of limited oxygen, polyphenols can be oxidized non-enzymatically [82]. The polyphenols containing a catechol ring are oxidized to semiquinone and benzoquinone radicals (Figure 2). At the same time, the oxygen is reduced to H_2O_2, and the whole process is mediated by the redox cycle of Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} [83]. Recent reviews have illustrated the antimicrobial action of quinone and its derivatives [84–86]. It may be suggested that, in presence of ascorbic acid and transition metals, redox oxygen species may be generated, which may oxidize the polyphenols to corresponding quinones or benzoquinones, enhancing antimicrobial activity [87,88].

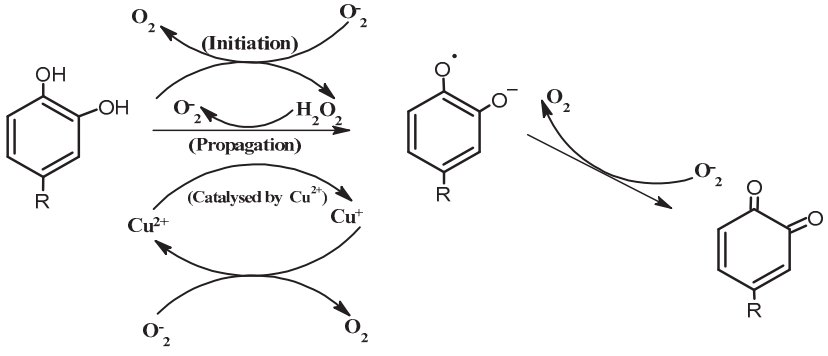

Figure 2. The figure illustrates the oxidation of the catechol ring containing polyphenols, which is oxidized to benzoquinone radical with an intermediate formation of semiquinone in presence of Cu^{2+} catalyst. The reaction starts with the initiation step of O_2 being converted to O_2^{-}, followed by a propagation step during which O_2^{-} is converted to H_2O_2 [89].

Phenolics from plants have been combined with other substances, sometimes referred to as adjuncts, such as transition metal ions or vitamin C to enhance phenolic efficacy [90,91]. Enhancement of antimicrobial activity was observed in the case of pomegranate rind Ext by Cu (II) alone or with both Cu (II) and AA combinations against many bacterial strains [92,93]. The addition of copper (II) sulfate and AA ascertained the enhancement of antimicrobial activities of whole and sub-fractionated white tea against *S. aureus* [94]. Enhanced antimicrobial activity against *S. aureus* and *E. coli* was also observed by the addition of the AA to the (+)-catechin–copper (II) mixture [95]. A recent study revealed

the antibacterial effect of AA against *S. enterica* subsp. *Enterica* serovar Typhi and *Vibrio fluvialis* could be enhanced when applied in a combination with linalool and copper [96]. However, the mechanism behind the enhancement in antimicrobial activity due to the addition of transition metals and AA to the plant polyphenols has not been appropriately elucidated yet.

4.2. Enhancement Using Degradation Products of Ascorbic Acid in an Ethanolic Solution

In aqueous (AQ) systems, AA is very unstable and efficiently degraded both aerobic (AB) and anaerobically. The degradation process of AA is complex and involves many oxidation–reductions and intermolecular rearrangement reactions. The degradation of AA via AB and anaerobic (AAB) pathways depends upon oxygen, heat, light, storage temperature, and time [97,98]. However, degradation of AA mainly results in the formation of volatile and brown products via self-degradation and non-enzymatic browning. The most commonly reported terminal products resulting from the AB degradation of AA and dehydroascorbic acid in acidic AQ conditions (pH 1–3), were found to be 3-hydroxy-2-pyrone and 2-furoic acid [99]. These are also amongst the highest yielding products depending on the conditions utilized. Heat-induced (60–100 °C) AB degradation of different solutions of AA and dehydroascorbic acid demonstrated that both 3-hydroxy-2-pyrone and 2-furoic acid were the main degradation products of AA (Figure 3A). This included decarboxylation of 2,3-diketogulonic acid with the formation of xylosone, a mechanism already reported by the authors of [100], followed by a multi-step conversion of xylosone to the terminal products via oxidation, dehydration, and/or ketoenol tautomerism [100]. Xylosone is a gateway to numerous degradation products, and its presence as an AA or dehydroascorbic acid degradation product has been confirmed by several studies [101,102].

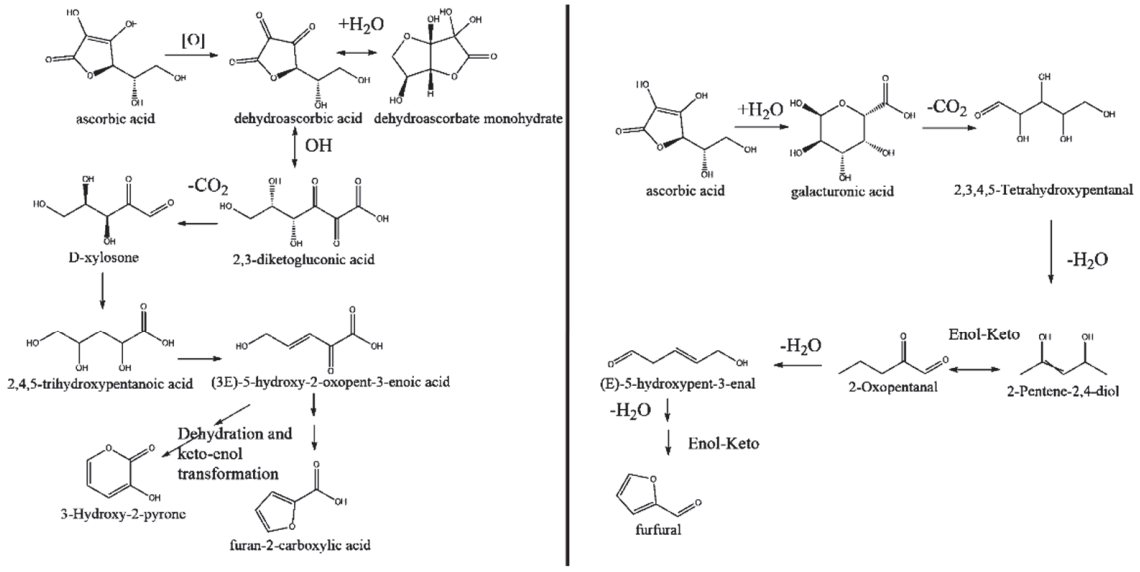

Figure 3. Mechanism of aerobic and anaerobic degradation of ascorbic acid. (**a**) aerobic degradation of ascorbic acid with the primary terminal product of degradation as 3-hydroxy-2-pyrone; (**b**) anaerobic degradation of ascorbic acid with the primary terminal product as furfural [103].

The term "AAB degradation" of AA refers to ascorbic acid degradation to some terminal product via a mechanistic pathway that does not require an oxidation step. The AAB degradation of AA does not generally require the removal of O_2 from a reaction system; however, the lowering of oxygen concentration has the advantage of limiting the competing AB reactions [103]. The degradation of AA by reaction with oxygen occurs faster than its AAB degradation [104]. However, the AAB degradation rate can increase considerably with higher temperatures [104]. The pH level is also known to influence the rate of AAB degradation of AA, which increases as the pH is raised from 2.3 to 4.0 [105].

Regardless, the most commonly reported terminal product for the AAB degradation of AA is furfural [99,106]. The general mechanism to describe the formation of furfural is shown in (Figure 3B) and involves initial ring cleavage and hydration of AA rather than oxidation [107]. The subsequent steps require decarboxylation, acid-catalyzed dehydration, and cyclization. Several studies have shown that the formation of furfural is favored at lower pH values [108,109].

Another mechanism has been reported for the acid-catalyzed AAB degradation of AA in methanol, forming a bicyclic structure similar to dehydroascorbic acid. It then undergoes dehydration and decarboxylation via dihydrofuran-type intermediates to afford furfural [110]. The authors proposed that the mechanism would be equally valid in AQ systems but did not provide evidence.

Not all scholars agree that furfural is exclusive to the AAB degradation of AA. Instead, it has been suggested that it can be formed via an oxidatively generated dehydroascorbic acid degradation pathway [111,112]. Another product unique to the AAB pathway is, 4,5-dihydroxy-2-ketopentanal (otherwise known as 3-deoxypentosulose) generated upon storage of AA at pH 3.5, although the storage temperature was not so relevant to wine conditions (120 °C for 2 h) [113]. This compound has been proposed as an intermediate in the formation of furfural during the AAB degradation of AA. The intermediate furan compounds which were generated during AA degradation have been found to inhibit the proliferation of *S. typhi* and *B. subtilis* to different extents [114]. The MIC values of furfural and furoic acid (terminal products of AA degradation) against *B. subtilis* and *S. typhi* were 0.027, 0.015, and 0.029, 0.009 µM, respectively [114]. Recent studies have reported antimicrobial action of furoic acid and furan compounds [115,116] The reported end product of AB and AAB degradation might have potency in enhancing the antimicrobial activity of plant Ext. However, to date, there is no evidence of the enhancement of the antimicrobial activity of plant Ext using the degradation product of AA.

4.3. Enhancement Using Maillard Reaction Products

The Maillard reaction (MR) is a heat-induced browning reaction widely employed in various fields in the food industry that has been used as an effective method for protein modification and the production of remarkable changes in the structure and bioactivity of proteins [117,118]. In particular, MR products (MRPs) have been shown to have significant antibacterial activities against a wide range of bacteria, with lower toxicity than antibiotics [119,120]. The MRPs possess many intermediate products (Figure 4), such as aldehydes, ketones, and heterocyclic compounds, which can effectively inhibit the growth of some Gram-positive and Gram-negative bacteria [119].

MR products may have the potential to exhibit a synergistic antimicrobial effect in conjunction with phytochemicals from plant compounds, thus enhancing their potency (i.e., lowering the MIC value of the plant-derived compounds) [121]. The plant-derived polyphenols have been found to have additive, synergistic antimicrobial effects with the intermediate products of the MR such as diacetyls, carbonyls, and furfural [122,123]. Methylglyoxal (one of the intermediate products of MR) and catechin have been reported to positively affect antibacterial activity [124]. However, the antimicrobial activity of plant Ext or plant phytochemicals using MRPs is not yet reported, and further research is needed in this area.

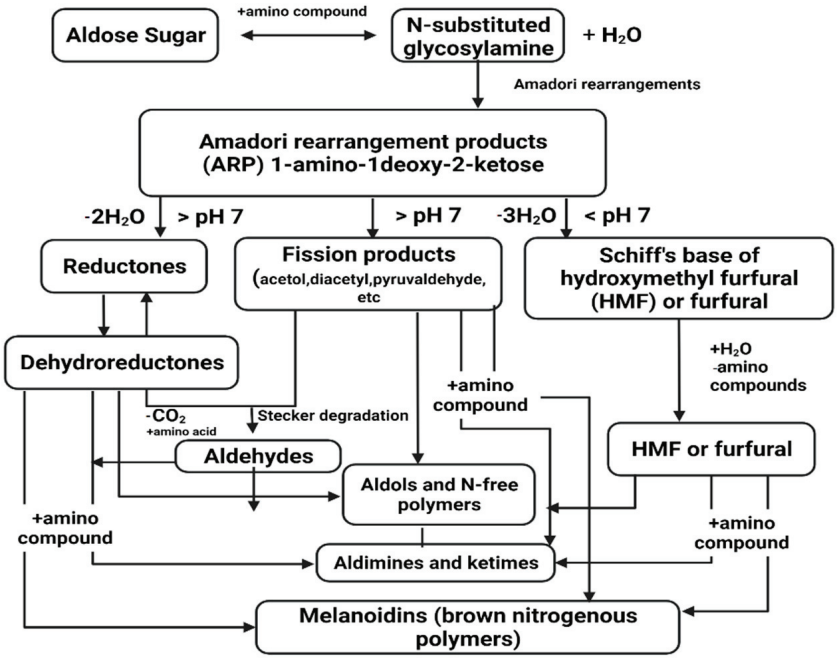

Figure 4. Scheme of Maillard reaction in different pH levels consisting of early stage with the formation of the ARP products and Schiff base, followed by the advanced stage, consisting of fission reaction, Stecker degradation, resulting in formation of advanced glycated products with a final stage of oxidation, condensation, cyclization, and rearrangement resulting in melanoidin polymers.

4.4. Enhancement Using Laccase–Mediator System

The oxidation of organic compounds to produce functionalized molecules is essential in organic synthesis [125]. Controlled enzymatic oxidation or hypo-oxidation can yet be another approach toward enhancing the antimicrobial activities of phytocompounds. Increased bioactivities have been observed by the biochemical transformation of triterpenes using oxidative enzymes [126]. Oxidoreductive enzymes such as laccase and peroxidise can transform phenols through oxidative coupling reactions with the production of polymeric products by self-coupling or cross-coupling with other molecules.

Laccase (EC 1.10.3.2), is a multi-copper oxidase that couples the four-electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenols, methoxy-substituted phenols, anilines, aryl diamines, hydroxyindoles, benzenethiols and inorganic/organic metal compounds by a one-electron transfer mechanism, making this green enzyme useful for carrying out several types of oxidative reactions [127–132]. Laccases use O_2 as the electron acceptor to remove protons from the phenolic hydroxyl groups. This reaction gives rise to radicals that can spontaneously rearrange, which can either lead to the fission of C–C or C–O bonds of the alkyl side chains or the cleavage of aromatic rings [128]. The oxidation of a reducing substrate by laccase involves losing an electron and forming a free radical [129]. This radical is, in general, unstable and may undergo further laccase-catalyzed oxidation (e.g., quinone from phenol) or non-enzymatic reactions (e.g., hydration, disproportion, or polymerization) [132]. The electron transfer from the substrate to copper is controlled by the redox potential difference. The rate of substrate oxidation by laccase, which has high redox potential, is higher if it has a lower redox potential.

Enzymatic polymerization of phenolic compounds (catechol, resorcinol, and hydroquinone) has been carried out using laccase [133–135]. Intermediates (quinones) formation in the first stage of oxidation with further oxidation reaction, forming colored polymers,

was observed while evaluating the polymerization and the structures of the polymers by UV–Vis and Fourier transform infrared spectroscopy [134]. Changes in the color of flavonoids due to oxidation by the laccase enzyme were due to the polymerization and linkage of the quinones (Figure 5) formed as an intermediate [133]. Laccase oxidation of caffeic acid and isoeugenol was shown to enhance their antimicrobial activity against *S. aureus* and *E. coli* in liquid media [135]. Some low molecular weight phenolic compounds are usually produced as a result of oxidative metabolism by C ring cleavage of catechin and epicatechins [136]. The antimicrobial properties of one of these low molecular weight polyphenols, 3,4 dihydroxy benzoic acid have been well established [137,138]. Moreover, dimers and polymers of flavonoids have also been found to have superior antimicrobial effects in comparison to the parent monomer [139–141]. It has been suggested that the toxicity of the laccase-treated olive Ext can be due to the presence of phenolic compounds such as ortho-benzoquinones, quinonoid, or oxidative coupling polymers, which results because of Lac treatment is more toxic than the parent compounds [142]. Many studies have suggested polymerization of the phenolics using the Lac enzyme [143,144]. Enhanced antimicrobial activity of the resulting oligomers and polymers has also been reported [145,146].

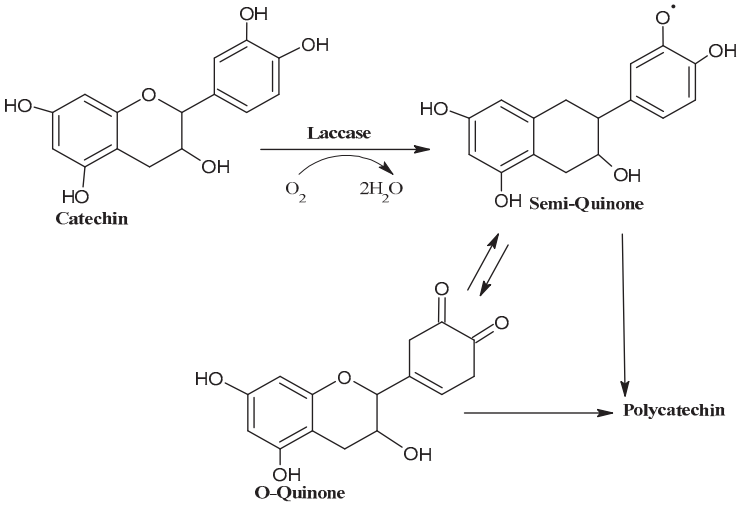

Figure 5. Mechanism of catechin oxidation by laccase where catechin is polymerized into polycatechin with the intermediate formation of semiquinone radicals and orthoquinone.

Although laccase can oxidize a wide range of substrates, some substrates of interest cannot be oxidized directly by laccases, either because they are too large to fit into the enzyme active site or because they have an exceptionally high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of so-called "chemical mediators", which are suitable compounds that act as intermediate substrates for the laccase, whose oxidized radical forms can interact with the bulky or high redox-potential substrate targets [131]. Laccase–mediator system (Figure 6) has found its immense application in the degradation of lignin. Small redox molecules such as 3-hydroxyanthranilic acid (HAA) might act as "electron shuttles" between the enzyme and lignin and cause polymer de-branching and degradation [131]. Some examples of laccase mediators extensively used are 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), N-hydroxybenzotriazole (HBT), N-hydroxyphtalimide (HPI), violuric acid (VLA), N-hydroxyacetanilide (NHA), methyl ester of 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid), and 2,2,6,6-tetramethyl piperidine-1-yloxy (TEMPO) [131].

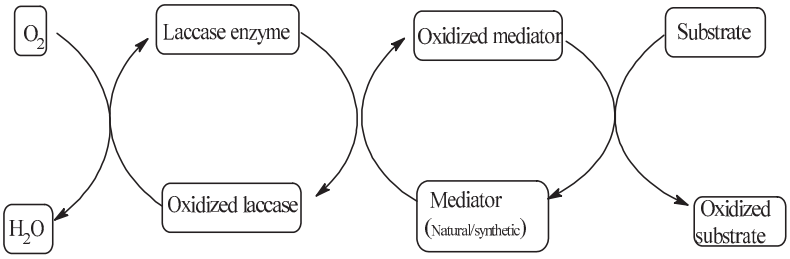

Figure 6. Schematic representation of laccase-catalyzed redox cycles according to which laccase is oxydize in presence of oxygen, itself oxidizing the mediator, and the oxidized mediator further initiates the oxidation of the substrate, with high redox potential [131].

Laccase is capable of oxidizing unreactive iodide to reactive iodine [147]. Phenolic compounds such as vanillin, which resembles substructures of softwood lignin, can be directly iodinated by reacting with laccase and iodide, resulting in compounds with antifungal activity [147]. The addition of redox mediators "acetosyringone" in catalytic concentrations increased the rate of iodide oxidation by ten-fold and the yield of iodo-vanillin by 50% [147].

Functionalization is the process of adding new functions, features, capabilities, or properties to material by changing the surface chemistry of the material [148]. In one study, functionalization of chitosan with phenolic acids such as caffeic acid or gallic acid using laccase from *Trametes versicolor* formed a product with enhanced antimicrobial activity against *E. coli* and *L. monocytogenes* [149]. The authors proposed the functionalization of chitosan with the phenolic acids by laccase catalyzed oxidation of phenolic acids to electrophilic o-quinones, which undergo a new oligomer/polymer-forming structure originated by C–C coupling between the benzene rings and C–O–C coupling involved with the phenolic side chains.

4.5. Enhancement Using Peroxidase Enzyme

The peroxidases (EC 1.11.1.7) are heme proteins and contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. They come under the class of oxidoreductases that catalyze the oxidation of a wide range of molecules, using peroxide as an electron acceptor [150]. The reduction of peroxides at the expense of electron-donating substrates makes peroxidases useful in several industrial and analytical applications. The common overall reaction of the peroxidases can be written as in the following Equation (3), where RH is a suitable peroxidase substrate and R is a free-radical product derived from it as follows:

$$2RH + H_2O_2 \rightarrow 2R^{\bullet} + 2H_2O \quad (3)$$

Oxidation reactions carried out by peroxidase may be one-electron or two-electron oxidation. A classic example of one-electron oxidation is guaiacol assay through which guaiacol is oxidized to a free radical that undergoes a subsequent radical–radical combination to give a colored dimeric product. The dimerization can occur between two ring carbon atoms or by adding the oxygen of one phenoxy radical to the ring carbon of the other [151]. Two-electron oxidation is rare for most peroxidase enzymes. The example under this type is the oxidation of halide and pseudo-halide ions, specifically I^-, Br^-, Cl^-, and NCS^-. The oxidation of I^- and NCS^- is common for the peroxidases [152]. Many reactions are known in which oxidation of a substrate by peroxidases produces a two-electron oxidized product. These reactions can be rationalized by forming a free radical metabolite, followed by the second oxidation of the free radical to the final observed product.

Lactoperoxidase, together with thiocyanate ions and hydrogen peroxide generates, hypothiocyanite ions and the oxidized product which is known as the lactoperoxidase system. The oxidized effect possesses a broad spectrum of antimicrobial activity. Hence,

much attention has been paid to the lactoperoxidase system [153], e.g., lactoperoxidase systems mediated by oxidized β-carotene/SCN^- cycling lead to enhanced antimicrobial effects [154].

4.6. Future Perspective

Structural changes in polyphenols by biochemical modifications can be elucidated through elemental analysis and spectral data (IR, ^{1}H NMR). Furthermore, crystal, and molecular structures of the potential metal flavonoid or phenolic acid complexes can be identified by using single-crystal X-ray diffraction data. By electron paramagnetic resonance spectroscopy, transient oxidation species could also be identified. Morphological study of the bacterial and fungal cells triggered by biochemically modified polyphenols can reveal the possible antimicrobial effect on the cells. Membrane potential study may indicate the mechanism by which the antimicrobials could have affected the membrane permeability. Ultimately, antibacterial effects against food pathogens can be carried out to understand the scope of biochemically formulated antimicrobials in food applications.

5. Conclusions

Polyphenols are widely and easily available bioactive compounds that have health benefits. Despite their benefits, their use in food applications is limited, due to their low potency and high concentration needs. This review showed different biochemical means of improving the antimicrobial property of plant-based polyphenols. Considering the structural importance of the antimicrobial properties of polyphenols, this report also showed the significance of different classes of polyphenols as active antimicrobial agents. Although the mechanisms of the biochemical methods involved in enhancing the antimicrobial activity of plant polyphenols have been explained in this review, the possible structural and functional modifications that these biochemical methods may bring about in the modified polyphenols have not yet been established completely. Therefore, further validation of these methods through high-throughput techniques is crucial.

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Review

Volatile Organic Compounds from Basil Essential Oils: Plant Taxonomy, Biological Activities, and Their Applications in Tropical Fruit Productions

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Abstract: Basils of the genus *Ocimum* are aromatic plants grown widely throughout the tropical and temperate regions. The essential oils obtained from their aerial parts are enriched with volatile organic compounds with high market demand for food and pharmaceutical industries. The volatile organic compounds have been shown to exhibit biological activities. Therefore, their novel applications have been extensively explored in the last few decades. The most widely available basils in the tropical areas include white holy basil (*O. sanctum* var. Shyama), red holy basil (*O. sanctum* var. Rama), Thai basil (*O. basilicum* var. thyrsiflorum), lemon basil (*O. citriodorum*), and tree basil (*O. gratissimum*). Over 60 volatiles of different classes have been exclusively described, and some of them could be useful as biomarkers for genotype specification. The major volatile ingredient is the phenylpropanoids, such as methyl eugenol, which has the potential as a natural product for mitigating Oriental fruit fly (*Bactrocera dorsalis*) during tropical fruit production. Moreover, basil essential oils are also used to control diseases of the fruits during post-harvest storage. As a result, the application of basil essential oils as a sustainable defect control strategy for tropical fruit value chains seems intriguing. This review provides comprehensive information on plant taxonomy and volatile compositions of the essential oil fractions from different basil species. Their biological activities and applications are also discussed, mainly during the pre- and post-production of tropical fruits. Additionally, the available techniques to enhance the efficacy of the volatile active compounds are also described.

Keywords: essential oil; integrated pest management; Oriental fruit fly; pomology; post-harvest disease control

1. Introduction

Ocimum is one of the important genera within the wealthiest essential oil-bearing plant family, the Lamiaceae. It is represented by more than 150 species cultivated and distributed throughout the tropical and temperate regions [1]. They are collectively known as the "basils" that retain the commercial demand for their nutritional, aromatic, ornamental, culinary, religious, and medicinal importance [2]. Different basil types are commonly used, including holy basil (*O. sanctum*), sweet or Thai basil (*O. basilicum*), lemon basil (*O. citriodorum*), and tree basil (*O. gratissimum)* [3,4]. It is well established that different basil cultivars have the genetic potential to create and maintain distinct sets of volatile components, resulting in a wide variety of chemotypes within the same basil species [5]. The essential oils of these basils are predominantly constituted of phenylpropanoids such as estragole, eugenol, and methyl eugenol; however, they also contain common monoterpenes such as geranial, neral, and α-ocimene, as well as sesquiterpenes such as β-caryophyllene, α-cubebene, and γ-muurolene [6]. Most of which are biologically active on living organisms, especially the antimicrobial and antioxidant properties for food and medicinal uses [7,8]. It was discovered that eugenol has antimicrobial and analgesic effects on humans [9]. Additionally, the essential oils also possess a wide range of biological functions that theoretically minimise post-harvest deteriorations. Volatile organic compounds have been shown to inhibit the growth of microorganisms, especially those responsible for post-harvest diseases such as *Aspergillus* spp. [10–12], *Colletotrichum acutatum* [13], *Botrytis cinerea* [14], and *Penicillium italicum* [15]. They have also been extensively used in insect pest management to control rice weevil (*Sitophilus oryzae*) [16], bean weevil (*Acanthoscelides obtectus*) [17], and cotton bollworm (*Helicoverpa armigera*) [18]. Prominently, methyl eugenol has been claimed for its ability to attract Oriental fruit flies (*Bactrocera dorsalis*) [19], the most important tropical fruit pest [20,21]. The estimated annual loss from this pest alone is roughly over US$ 100 million, and mangoes have been the most susceptible crops [20,21]. Aside from the infestation of the Oriental fruit flies that cause physiological damage to fresh fruits, biological stress could encourage post-harvest biochemical mechanisms such as browning and physiological decay [22]. A study of fresh apple has also proven that spraying the sweet basil essential oil on the fruit skin illustrated the preservative effect, thereby extending its shelf life [23]. With all these advantages, it is interesting to use basil essential oils as biological controls during the production of tropical fruits. However, the instability of essential oils at ambient conditions, as well as harsh environmental exposure, are the limitations. Moreover, volatile organic compounds decompose quickly with the presence of light, heat, humidity, and oxygen [24].

This review aims to serve as a guide to using the volatile components obtained from commercially available *Ocimum* species in the development of functional products for the sustainable production of tropical fruits. It attempts to provide the relevant data, both taxonomical and chemotypes, with particular attention to the biological activities and applications. The typical constraints of these applied uses are discussed, along with the recent approaches to improve efficiency.

2. Taxonomy

The genus *Ocimum* is known as one of the most prominent genera in the Lamiaceae family and currently comprises more than 150 species [1,25]. The distribution is mainly in the tropical and template regions and is likely to have originated (mainly the holy basil) in India [26]. Recently, they have been cultivated worldwide as culinary herbs and for essential oil extraction [27]. Taxonomical identification within the genus and between the varieties can be made by the morphological characteristics such as leaf shape and its colour, flower, and seed morphology [4,28]. Numerous polymorphisms resulting from extended cultivation and inter- and intra-specific cross hybridisation result in a vast range of subspecies, each with its own chemical makeup and biological activity [25,29,30].

The commonly available *Ocimum* plants were studied in the previous work for their distinct morphological characteristics [6]. The leaf is generally simple, petiolate and the

leaf blade is ovate with a rounded base, oblique, and the apex is acute. *O. gratissimum* has a large leaf size (~45 cm^2), whereas *O. citriodorum* has a leaf size of around 3.5 cm^2 (Figure 1c,d). The *O. sanctum* of var. Rama and Shyama can be distinguished by having the aerials of red and white (Figure 1a). Similarly, different leaf and stem colours were noticed, ranging from red, purple-green, and green among the different varieties of the *O. basilicum* L. used in Iran [31]. Singh [32] used the number of leaf veins to show that *O. americanum* was described to have seven distinct veins, and the mid-vein reached the apex, while *O. tenuiflorum* has nine distinct veins, and the mid-vein does not reach the apex. The typical inflorescence of *Ocimum* spp. is a thyrse composed of opposite 1–3-flowered cymes (Figure 1b) [33]. The calyx is generally a short tube or funnel-shaped; it is straight or slightly curved. The corolla is formed forward (sometimes bent downwards), larger upper lip and a smaller lower one and declinate stamens [34]. The posterior lip of the corolla comprises four lobes. There are always four stamens, an anterior pair that attaches near the corolla mouth and a posterior pair that connects close to the corolla base. The size of basil seeds varies depending on the phenotype, cultivating location, and moisture content [35]. Its colour can occasionally be used to differentiate between varieties [36]. The complex polysaccharide structure gives the seed a unique mucilaginous characteristic after soaking in water, which is prominent in *O. citriodorum* and *O. basilicum* var. thyrsiflorum. Table 1 illustrates the taxonomical characteristics of different basil species.

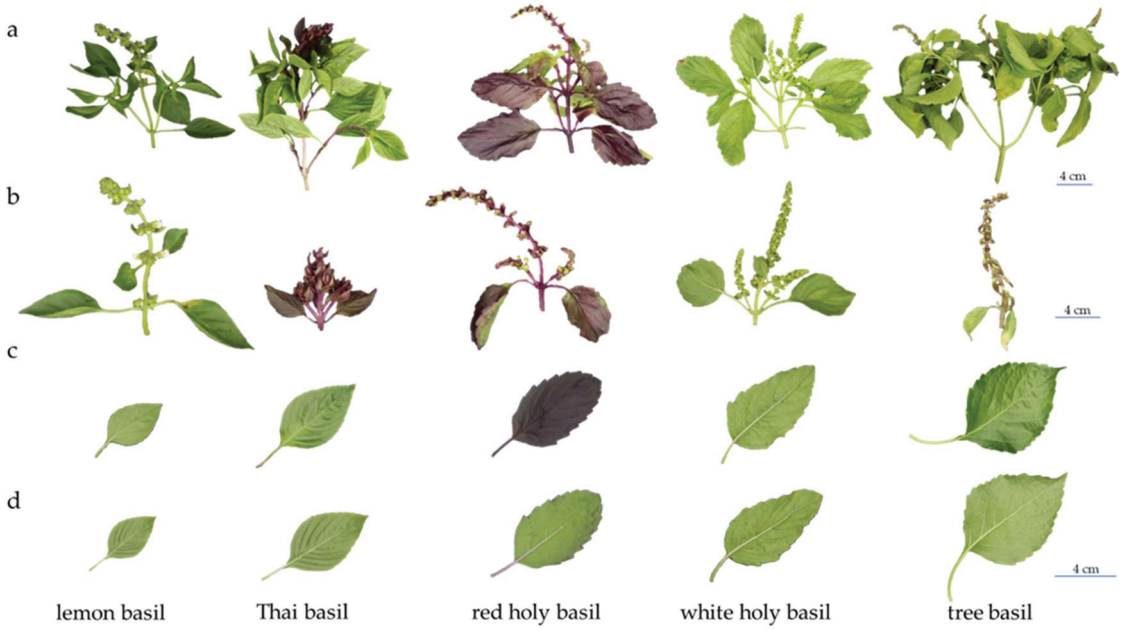


Figure 1. Morphological characteristics of some *Ocimum* species; aerial part (**a**) inflorescence (**b**) upper (**c**) and lower (**d**) leaf surface of lemon basil (*O. citriodorum*), Thai basil (*O. basilicum* var. thyrsiflorum), red holy basil (*O. sanctum* var. Rama), white holy basil (*O. sanctum* var. Shyama) and tree basil (*O. gratissimum*).

Table 1. Comparison of morphological characteristics of studied *Ocimum* species.

Scientific Name	Common Name	Overall Characteristic	Leaf		Fluorescence		Seed Characteristics	Location	References
			General Characteristic	Leaf Colour	General Characteristic	Flower Colour			
O. americanum L.	bon tulsi	annual, herb, 20–60 cm tall	leaf elliptic-lanceolate, leaf surface glabrous except hairy midrib, veinlets, and margin	grey green	inflorescence greenish, calyx green with sometimes purplish stripe, long hairy	white	seed black, narrowly ellipsoid, mucilaginous	India	[25]
O. × *africanum Lour.* or *O. citriodorum*	lebu tulsi, lemon basil	annual, herb, 45–105 cm tall	leaf size ~3.5 × 1 cm, leaf elliptic—broadly obovate, glabrous except hairy midrib, veinlets, and margin	n/d	inflorescence greenish, calyx green, long hairy	white	seed brownish-black, ellipsoid, mucilaginous	India, Thailand	[6,25]
O. basilicum var. thyrsiflorum	Thai basil, marua tulsi	annual, herb, 45–100 cm tall	leaf size ~5.5 × 2 cm, leaf ovate-lanceolate to oblong-lanceolate, glabrous except hairy midrib, veinlets, and margin	n/d	inflorescence greenish, calyx green, long hairy	pinkish white	seed brownish-black, ellipsoid, mucilaginous	Thailand, India	[6,25]
O. gratissimum var. *macrophyllum*	tree basil, clove basil, African basil, ram tulsi	perennial, undershrub, or shrub, 140–200 cm tall	leaf size ~9 × 5 cm, leaf lanceolate, ovate or ovate-lanceolate, glabrous except hairy midrib	n/d	inflorescence greenish-purple, calyx greenish-purple, hairy	purple, yellowish-white	seed brown, sub-globose, non-mucilaginous	Thailand, India	[6,25,37,38]
O. kilimandscharicum Guerke	karpur tulsi	perennial, herb, 60–120 cm tall	leaf ovate-oblong, leaf surface pubescent with white hairs on both sides, much denser and longer on veins beneath	n/d	inflorescence, greenish-greyish, calyx greenish-greyish, densely hairy	white	seed black, narrowly ellipsoid, mucilaginous	India	[25]
O. sanctum or *O. tenuiflorum* var. Shyama	red holy basil, krishna tulsi	annual to biannual, branched sub-shrub 30–150 cm tall	simple opposite leaves, leaf size ~4 × 1.5 cm, ovate-obovate, elliptic-oblong, surface patently hairy to clothed with soft spreading hair, purple leaf	purple	inflorescence purple, calyx purple, patently hairy to densely pubescent	purplish	seed brown, globose, non-mucilaginous	Thailand, India	[6,25,39]
O. sanctum or *O. tenuiflorum* var. Rama	white holy basil, radha tulsi	annual to biannual, branched sub-shrub 30–160 cm tall	simple opposite leaves, leaf size ~4 × 1.5 cm, leaf ovate-obovate, elliptic-oblong, surface patently hairy to clothed with soft spreading hair, green leaf	green	inflorescence green-greenish-purple, calyx green, patently hairy to densely pubescent	purplish	seed brown, globose, non-mucilaginous	Thailand, India	[6,25,39]
O. suave or *O. gratissimum* var. *suave*	holy basil, wild basil	92.75 cm tall, 84.42 cm width	leaf blade ovate-oblong to oblong ~5–12 × 1.5–6 cm, gradually reduced toward apex, slightly scabrid	grey green	inflorescence with persistent bracts, calyces flattened dorsoventrally tinged with brown, corolla small white	white	brownish, black-globose, subglobose, non-mucilaginous	India	[38,40–42]
O. viride	African basil, nunum	158.58 cm. tall, 114.71 cm width	leaf size ~4.73 × 9.46 cm	n/d	less conspicuous, autogamous, fruiting calyx large amount of terpene	brownish green	brownish, black-globose, subglobose, non-mucilaginous when wetted	India	[38,40,43,44]

n/d = no data.

3. Volatile Chemical Compositions of Basil Essential Oils

A number of unique epidermal structures known as trichomes are developed on the surface of the aerial part, which may or may not be secretory [45]. These include the glandular trichomes where the essential oil is localised and the non-glandular trichomes for pest defence [46]. Essential oils are refined lipophilic mixes derived as liquids that possess aromatic properties due to the volatile aroma-active components (i.e., molecules that elicit a distinctive taste and smell) [47]. According to the French Agency for Normalization (AFNOR), the essential oil is defined as follows (NF T 75-006): "The essential oil is the product obtained from a vegetable raw material, either by steam distillation or mechanical processes, from the epicarp of citrus, or dry." [48]. The conventional essential oil extractions

are steam distillation [49,50] and hydro-distillation [51,52]. However, a few techniques have been used to enhance the efficiency of the extraction process, including microwave-assisted extraction [52,53] and ultrasonication [51]. The extraction techniques and processes used to influence the quality and quantity of the extract result in a range of bioactive levels, for example, biopesticide activity against stored-grain pests [51]. Basil plants contain up to 1% of the essential oil, depending on genotypes, cultivation, growing location, and post-harvest management [54–57]. The essential oils are more concentrated in leaves and flowers and much less in the stems [58]. In the study of different basils used as culinary herbs, the essential oil yield of white holy basil (*O. sanctum* var. Rama) and Thai basil (*O. basilicum* var. thyrsiflorum) was ~0.4%, followed by lemon basil (*O. citriodorum*) and red holy basil (*O. sanctum* var. Shyama) ~0.3%, and tree basil *O. gratissimum*) was the least (<~0.2%) [6]. Variation of essential oil colours also depends on the genotypes, harvesting stages as well as different extraction techniques [58,59]. Under the visible light, the essential oil of *O. gratissimum*, *O. citriodorum*, *O. sanctum*, and *O. basilicum* var. thyrsiflorum colour are orange, yellow, and colourless, respectively. However, the colour difference is not noticed within the same species, such as those of white and red holy basils (*O. sanctum* var. Rama and Shyama) [6,58,60]. According to this, the volatile chemical compositions of essential oils may play a crucial role in the colour characteristic of the essential oils [61]. Other factors include thermal degradation, oxidation, isomerisation, dehydrogenation, and polymerisation [62–64].

Essential oils are a complex mixture of various classes of volatile organic components such as alcohols, aldehydes, esters, ketones, phenylpropanoids, and terpenoids [65]. Table 2 illustrates the different volatile classes in the essential oils with the representative descriptors of the *Ocimum* plants. The essential oil profiles are displayed by the heat map of mass spectrums of the different volatile components from Thai basil plants (Figure 2). It is apparent that there is the closest relationship between the volatile organic compounds of plants within the same species (white and red holy basils). The phenylpropanoids (estragole, eugenol, and methyl eugenol) are dominant with a proportion of up to 30–50% of analysed compounds, followed by the sesquiterpenes (i.e., trans-caryophyllene, trans-α-bergamotene, τ-cadinol, cis-α-bisabolene, β-elemene, and germacrene) and monoterpenes (i.e., trans-ocimene, linalool, 1,8-cineole, and camphor) [57,65,66]. The principal constituents of *O. citriodorum* essential oil are estragole, citral, and neral, which serve as crucial fingerprints representing its distinctive citrus scent [6]. Holy basil oil comprises a mixture of 17 volatile compounds with methyl eugenol, trans-caryophyllene, eugenol representing clove-like aroma being dominant [6,66,67]. In the essential oil of *O. basilicum*, estragole is the key volatile element. At the same time, others, such as those of alcohols (i.e., linalool), ketones (i.e., camphor), and esters, are variable among different varieties [68]. It also illustrates that *O. gratissimum* essential oil is enriched with eugenol, trans-ocimene, trans-α-bergamotene, and linalool as the significant components [6,66] projected away from the other basil species. In another study, thymol, eugenol, and geraniol were used as volatile markers to distinguish sub-varieties grown in the USA [69].

Table 2. Chemical classes of the volatile organic compounds in the essential oils of the *Ocimum* spp.

No.	Volatile Organic COMPOUNDS	Odour Type	Odour Description	Chemical Class	*Ocimum* Species [7,8]
1	3-hexen-1-ol	green [1]	fresh, green, cut grass, foliage, vegetable, herbal, oily [1]	alcohol	TrB
2	1-octen-3-ol	earthy [1]	mushroom, earthy, green, oily, fungal, raw, chicken [1]	alcohol	LB, TrB
3	3-octanol	earthy [1]	earthy, mushroom, herbal, melon, citrus, woody, spicy, minty [1]	alcohol	Trb
4	linalool	floral [1]	citrus, floral, sweet, woody, green, blueberry [1]	alcohol	LB, RB, TB, TrB, WB

Table 2. *Cont.*

No.	Volatile Organic COMPOUNDS	Odour Type	Odour Description	Chemical Class	*Ocimum* Species [7,8]
5	borneol	balsamic [1]	pine, woody, camphor [1]	alcohol	RB, WB
6	terpinen-4-ol	spicy [1]	peppery, woody, earthy, musty, sweet [1]	alcohol	LB, TB
7	l-borneol	balsamic [1]	pine, woody, camphoreous, peppery [1]	alcohol	RB, WB
8	p-mentha-1,5-dien-8-ol	n/d	n/d	alcohol	TrB
9	fenchol	camphoreous [1]	camphoreous, pine, woody, dry, rooty, sweet, lemon [1]	alcohol	LB, TB, WB
10	(e,e)-2,6-dimethyl-3,5,7-octatrien-2-ol	n/d	n/d	alcohol	TrB
11	nerol	floral [1]	sweet, natural, neroli, citrus, magnolia [1]	alcohol	LB
12	geraniol	floral [1]	sweet, floral, fruity, rose, waxy, citrus [1]	alcohol	LB
13	elemol	spicy [1]	spicy, citrus, woody, resinous [1]	alcohol	RB, WB
14	spathulenol	earthy [1]	earthy, herbal, fruity [1]	alcohol	TB, TrB
15	(z)-4-decen-1-ol	waxy [1]	waxy, fatty, fruity [1]	alcohol	RB
16	lanceol	n/d	n/d	alcohol	TrB
17	cubenol	spicy [1]	spicy, herbal, green tea [1]	alcohol	TB
18	τ-cadinol	balsamic [1]	balsamic, earthy [1]	alcohol	LB, TB, WB
19	β-eudesmol	woody [1]	woody, green [1]	alcohol	LB
20	α-cadinol	herbal [1]	herbal, woody [1]	alcohol	TrB
21	juniper camphor	n/d	n/d	alcohol	RB, WB
22	α-bisabolol	floral [1]	floral, peppery, balsamic, clean [1]	alcohol	LB
23	(e)-hex-2-enal	green [1]	green, banana, aldehydic, fatty, cheesy [1]	aldehyde	TrB
24	trans-chrysanthemal	n/d	n/d	aldehyde	TB
25	neral	citrus [1]	sweet, citrus, lemon, lemon peel [1]	aldehyde	LB
26	geranial	n/d	pleasant citrus [6]	aldehyde	LB
27	citral	citrus [1]	sharp lemon, sweet [1]	aldehyde	LB
28	estragole	anisic [1]	sweet, sassafras, anise, spicy, green, herbal, fennel [1]	benzene derivative, ether	LB, RB, TB, WB
29	methyl eugenol	spicy [1]	sweet fresh, warm spicy, clove, carnation, cinnamon [1]	benzene derivative, ether	LB, RB, TB, TrB, WB
30	eugenol	spicy [1]	sweet, spicy, clove, woody [1]	benzene derivative, ether, alcohol	LB, RB, TrB, WB
31	1-bromo-8-heptadecyne	n/d	n/d	bromoalkene	LB
32	methyl 2-methylbutanoate	fruity [1]	etherial, iifting, fruity, tutti-frutti and ripe with a fatty, green nuance [1]	ester	TrB
33	bornyl acetate	balsamic [1]	woody, pine, herbal, cedar, spicy [1]	ester	LB
34	1,8-cineole	herbal [1]	eucalyptus, herbal, camphoreous, medicinal [1]	ether	LB, TB
35	trans-epoxyocimene	n/d	n/d	ether	TrB
36	nerol oxide	green [1]	green, weedy, cortex, herbal, narcissus, celery [1]	ether	LB

Table 2. *Cont.*

No.	Volatile Organic COMPOUNDS	Odour Type	Odour Description	Chemical Class	*Ocimum* Species [7,8]
37	caryophyllene oxide	spicy [1]	sweet, fresh, dry, woody, spicy [1]	ether	LB, RB, TrB, WB
38	humulene epoxide ii	n/d	n/d	ether	LB, TB
39	ledene oxide-(ii)	n/d	n/d	ether	TrB
40	6-methyl-5-hepten-2-one	citrus [1]	citrus, green, musty, lemongrass, apple [1]	ketone	LB, TrB
41	fenchone	n/d	eucalyptus-like, mouldy [2]	ketone	LB
42	camphor	camphoreous [1]	camphoraceous [3]	ketone	LB, TB, WB
43	6-methyl-hepta-3,5-dien-2-one	spicy [1]	cinnamon, coconut, spicy, woody, sweet, weedy [1]	ketone	TrB
44	salvial-4(14)-en-1-one	n/d	n/d	ketone	TrB
45	α-pinene	herbal [1]	fresh, camphoreous, sweet, pine, earthy, woody [1]	monoterpene	WB
46	β-pinene	herbal [1]	dry, woody, resinous, pine, hay, green, eucalyptus, camphoreous [1]	monoterpene	LB, TB, WB
47	camphene	woody [1]	woody, herbal, fir, needle [1]	monoterpene	WB
48	myrcene	spicy [1]	peppery, terpenic, spicy, balsamic, plastic [1]	monoterpene	TB, TrB
49	α-ocimene	fruity [1]	fruity, floral, cloth, laundered cloth [1]	monoterpene	TB, TrB
50	l-limonene	terpenic [1]	terpenic, pine, herbal, peppery [1]	monoterpene	TB
51	γ-terpinene	terpenic [1]	oily, woody, terpenic, lemon/lime, tropical herbal [1]	monoterpene	LB, TB
52	β-ocimene	floral [1]	citrus, tropical green, terpenic, woody, green [1]	monoterpene	LB, TrB
53	3-carene	citrus [1]	citrus, terpenic, herbal, pine, solvent, resinous, phenolic, cypress, medicinal, woody [1]	monoterpene	RB, TB, TrB
54	(e)-3,7-dimethylocta-1,3,6-triene	herbal [1]	sweet, herbal [1]	monoterpene	TB
55	(3e,5e)-2,6-dimethyl-1,3,5,7-octatetraene	n/d	n/d	monoterpene	TrB
56	3-methyl-1,4-heptadiene	n/d	n/d	monoterpene	TB
57	2,6-dimethyl-2,4,6-octatriene	floral [1]	sweet, floral, nut, skin, peppery, herbal, tropical [1]	monoterpene	TrB
58	(r)-α-pinene	n/d	n/d	monoterpene	TB
59	(+) -(−)-3-carene	citrus [1]	sweet, turpentine-like [1]	monoterpene	TB
60	α-copaene	woody [1]	woody, spicy, honey [1]	sesquiterpene	LB, RB, TB, WB
61	β-bourbonene	herbal [1]	herbal, woody, floral balsamic [1]	sesquiterpene	TrB
62	β-cubebene	citrus [1]	citrus, fruity, radish [1]	sesquiterpene	RB, TrB
63	β-elemene	herbal [1]	herbal, waxy, fresh [1]	sesquiterpene	LB, RB, TB, WB
64	caryophyllene	spicy [1]	sweet, woody, spicy, clove, dry [1]	sesquiterpene	LB, RB, TrB, WB
65	α-bergamotene	woody [1]	woody, warm, tea [1]	sesquiterpene	LB, TB, TrB
66	(z,e)-α-farnesene	n/d	n/d	sesquiterpene	TrB
67	rotundene	n/d	n/d	sesquiterpene	RB
68	α-guaiene	woody [1]	sweet, woody, balsamic, peppery [1]	sesquiterpene	LB

Table 2. *Cont.*

No.	Volatile Organic COMPOUNDS	Odour Type	Odour Description	Chemical Class	*Ocimum* Species [7,8]
69	β-sesquiphellandrene	herbal [1]	herbal, fruity, woody [1]	sesquiterpene	TrB
70	trans-α-bergamotene	woody [1]	woody, warm, tea [1]	sesquiterpene	LB, TB, TrB, WB
71	α-humulene	woody [1]	woody [1]	sesquiterpene	LB, RB, TrB, WB
72	bicyclo sesquiphellandrene	n/d	n/d	sesquiterpene	LB, TB, TrB
73	germacrene	n/d	spicy, woody [5]	sesquiterpene	LB, RB, TB, TrB, WB
74	trans-β-farnesene	floral [1]	floral, grass [4]	sesquiterpene	LB, TrB
75	γ-muurolene	woody [1]	herbal, woody, spicy [1]	sesquiterpene	TrB
76	β-selinene	herbal [1]	herbal [1]	sesquiterpene	LB, RB, WB
77	α-cubebene	herbal [1]	herbal, waxy [1]	sesquiterpene	RB, WB
78	α-selinene	herbal [1]	amber [1]	sesquiterpene	LB, RB, WB
79	bicyclogermacrene	greem [1]	green, woody, weedy [1]	sesquiterpene	LB, TB, TrB
80	α-bulnesene	n/d	n/d	sesquiterpene	TB, WB
81	β-gurjunene	n/d	n/d	sesquiterpene	RB
82	trans-α-bisabolene	n/d	n/d	sesquiterpene	LB, TrB
83	β-copaene	n/d	n/d	sesquiterpene	LB, TB, WB
84	δ-cadinene	herbal [1]	thyme, herbal, woody, dry [1]	sesquiterpene	LB, RB, TrB, WB
85	α-farnesene	woody [1]	citrus, herbal, lavender, bergamot, myrrh, neroli, green [1]	sesquiterpene	TrB
86	α-amorphene	n/d	n/d	sesquiterpene	ThB
87	α-amorphene	n/d	n/d	sesquiterpene	LB, WB
88	(z)-α-bisabolene	n/d	n/d	sesquiterpene	TB
89	eremophilene	n/d	n/d	sesquiterpene	RB
90	1,3-diisopropyl-1,3-cyclopentadiene	n/d	n/d	sesquiterpene	TrB
91	α-muurolene	woody [1]	woody [1]	sesquiterpene	RB
92	β-bisabolene	balsamic [1]	balsamic, woody [1]	sesquiterpene	LB

[1] The Good Scents Company Information System [70]; [2] Zeller and Rychlik [71]; [3] Pripdeevech et al. [72]; [4] Genovese et al. [73]; [5] Miyazawa et al. [74]; [6] Jiang and Kubota [75]; [7] Tangpao et al. [6]; [8] Tangpao et al. [66], LB = lemon basil (*O. citriodorum*); RB = red holy basil (*O. sanctum* var. Rama); TB = Thai basil (*O. basilicum* var. thyrsiflorum); TrB = tree basil (*O. gratissimum*); WB = white holy basil (*O. sanctum* var. Shyama), n/d = no data.

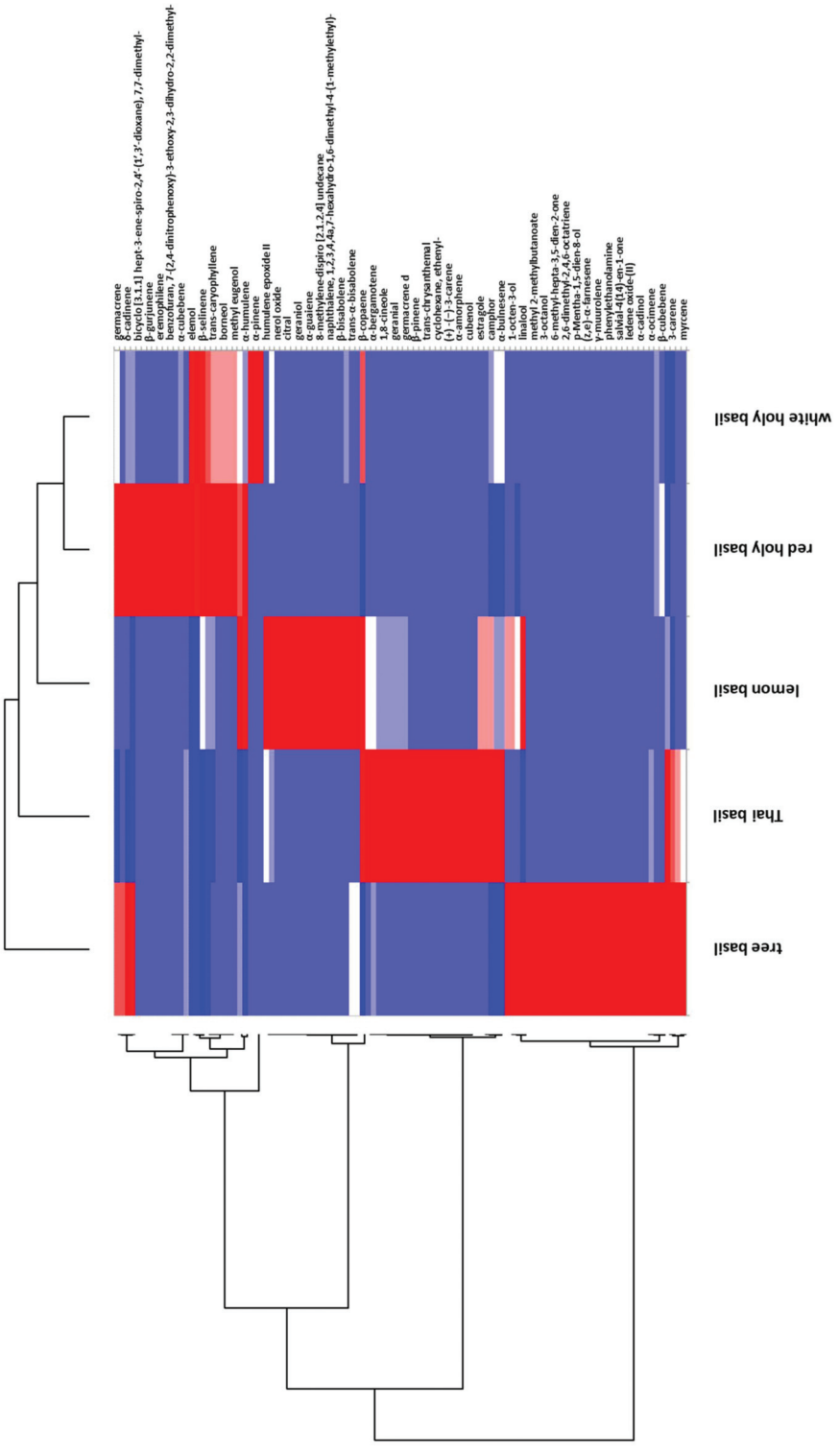


Figure 2. Heat map on volatile organic components in the essential oil of different basil species. The volatile components of different basil species as from the previous studies [6,66]. The heat map was generated using XLSTAT version 2020 (Addinsoft Inc., New York, NY, USA).

4. The Applications of the Basil Essential Oils in the Production of the Tropical Fruits

Plants synthesise various volatile organic compounds in the essential oils to defend themselves from environmental stresses, both biotic and abiotic [76]. These compounds' beneficial or adverse effects on the living matter are known as "biological activity". Consequently, essential oils have been used in many industrial applications and mainly replace synthetic chemicals [77–79]. During the pre- and post-harvest productions of the horticultural crops, essential oils were tested for their antipathogenic and pest control properties, such as insecticidal agents [80], repellents, attractants [81], and microbial disease controlling agents. In the production of most tropical fruits, the Oriental fruit flies attack the soft-skin fruits by laying eggs and feeding the larvae inside the fruits [66]. At post-harvest, *Colletotrichum* spp. is a major fungus causing anthracnose disease, accelerating the fruit deterioration process [82].

4.1. Pre-Harvest Applications

Several studies have investigated the control potential of essential oils from basil plants against pests during pre-harvesting (Table 3). The volatile organic constituents in the essential oils of some *Ocimum* species influence the behaviour of insects; for example, the ability to attract the Oriental fruit flies [66] and *Ceraeochrysa cubana* (herbivore predator) [83] as well as the repellent effect on *Allacophora foveicollis*, a serious pest that causes severe damage to pumpkin [84]. In addition, essential oils from *O. basilicum* and *O. gratissimum* were shown to have the ability to prevent egg hatching and adult emergence in *Callosobruchus maculatus*, the cowpea seed beetle [85]. Therefore, basil plants have been used as an intercrop in integrated pest management that has proven to reduce the total pest infestation in the cotton field [86] and greenhouse tomato production [87]. Methyl eugenol has been found in almost all types of basil essential oils, and it is the most active attractant for the Oriental fruit flies [66], while essential oil of the sweet basil is attractive to green lacewings *Ceraeochrysa cubana* Hagen (Neuroptera: Chrysopidae) [83]. The toxicity of methyl eugenol against larvae of the tobacco armyworms, *Spodoptera litura* has also been well defined [88]. Furthermore, the toxicity of *Ocimum* essential oils to fruit flies have been investigated [89]. Chang et al. [89] tested the toxicity of the three main components detected in the essential oil of *O. basilicum* L. viz., trans-anethole, estragole, and linalool. It successfully eliminated the flies, especially the estragole was the most effective.

The essential oil of sweet basil also illustrated the promising effect in controlling symptoms of wilt or root rot disease of cumin caused by *Fusarium* spp. [90]. It was also found that the mycelial growth of *Botrytis fabae* was significantly reduced by the basil oil types that were rich in methyl chavicol (or estragole) and linalool, while methyl chavicol, linalool, eugenol, and eucalyptol significantly reduced the overall growth of the fungus [10].

4.2. Post-Harvest Applications

In addition to their potential to control pre-harvest insect pests, the influence of extracts from *Omimum* spp. plants on the control of post-harvest insect pests was also investigated (Table 4). It was discovered that the essential oils of basils (*O. basilicum* and *O. tenuiflorum*) had volatile toxicity against stored-grain pests such as *Sitophilus oryzae*, *Rhyzopertha dominica*, *Cryptolestes* pusillus, *Sitophilus zeamais*, *Tribolium castaneum,* and *Acanthoscelides obtectus* [16,17,91–93] as well as the stored dates pests (*Ectomyelois ceratoniae* and *Ephestia kuehniella*) [94]. The powder form of the dried sweet basil plant has been used to repel *Sitophilus zeamais* Motschulsky, a post-harvest pest causing considerable damage to maise grain in most stores in Africa [95]. In addition to its ability to control insects, the role of essential oils as a natural post-harvest fungicide was also well recognised. From previous studies, the vapour of essential oils has the potential to inhibit post-harvest microorganisms [96,97], such as the harvested avocado fruit disease fungus (*Cercospora purpurea*) [98] and the peach and nectarine disease fungus (*Monilinia laxa*) [99]. The substances with low molecular weight and low polarity of essential oil play a role in the loss of cell membrane integrity of the pathogen by altering the pH in the cell, thereby inhibiting the growth as well

as inducing programmed cell death [100]. The essential oils from *Ocimum* spp. were able to inhibit the fungi causing the post-harvest diseases of the tropical fruits [10,13,14,101]. This also includes *Colletotrichum* spp., the fungus that causes anthracnose disease in common tropical fruits. Linalool is the most active substance in the *O. basilicum* essential oil that could inhibit the diseases of the stored seeds of lettuce and tomatoes caused by *F. oxysporum*, *Penicillium* spp., and *C. gloeosporioides* [102]. The crown rot pathogens that infected cut bananas during farm-level handling and packhouses were positively controlled by the combination of aluminium sulphate and basil oil in the modified atmosphere packaging during cold storage (12–14 °C) [103].

Table 3. Uses of volatile organic compounds from the studied *Ocimum* species against pests of horticulture crops.

Pests	*Ocimum* Species (Volatile Active Compounds)	Forms of Biological Activity	References
Bactrocera dorsalis (tropical fruit pest)	*O. sanctum* (methyl eugenol)	male fly attractant	[66]
Bactrocera dorsalis (tropical fruit pest)	*O. basilicum* (trans-anethole, estragole and linalool)	insecticide (100% mortality at 2 h after applying 10% oil)	[89]
Ceratitis capitata (fruit pest)	*O. basilicum* (trans-anethole, estragole and linalool)	insecticide (95% mortality at 2 h after applying 2.5% oil)	[89]
Bactrocera cucurbitae (tropical fruit pest)	*O. basilicum* (trans-anethole, estragole and linalool)	insecticide (100% mortality at 2 h after applying 7.5% oil)	[89]
Callosobruchus maculatus (cowpeas, green gram, and lentils pests)	*O. basilicum* and *O. gratissimum*	reducing egg hatch rate and the emergence of adults	[85]
Allacophora foveicollis (pumpkin pest)	*O. basilicum*	repellent	[84]
Botrytis fabae (cause of faba bean's chocolate spot disease)	*O. basilicum* (methyl chavicol, linalol, eugenol, and eucalyptol)	antifungal agent and fungicide	[10]
Uromyces fabae (cause of faba-bean rust)	*O. basilicum* (methyl chavicol, linalol, eugenol, and eucalyptol)	antifungal agent and fungicide	[10]
Fusarium spp. (cause of cumin root rot disease)	*O. basilicum* var. *basilicum* and var. *minimum)*	antifungal agent (antagonistic effect and reduction in mean disease rating of root rot in the in vivo test)	[90]

Table 4. Uses of volatile organic compounds from the studied *Ocimum* species during post-harvest management of horticultural produce.

Pests	*Ocimum* Species (Volatile Active Compound)	Forms of Biological Activity	References
Sitophilus oryzae (stored rice pest)	*O. basilicum* (methyl eugenol, estragole, linalool)	insecticide (30%–77% mortality at 24 h after fumigation of *O. basilicum* essential oil)	[93]
Rhyzopertha dominica (stored rice pest)	*O. basilicum* (methyl eugenol, estragole, linalool)	insecticide (37%–80% mortality at 24 h after fumigation of *O. basilicum* essential oil)	[93]

Table 4. *Cont.*

Pests	*Ocimum* Species (Volatile Active Compound)	Forms of Biological Activity	References
Cryptolestes pusillus (stored rice pest)	*O. basillicum* (methyl eugenol, estragole, linalool)	insecticide (90%–100% mortality at 24 h after fumigation of *O. basilicum* essential oil)	[93]
Ectomyelois ceratoniae (major insect pest of dates both in field and in storage)	*O. basilicum* (linalool, methyl cinnamate, and eugenol)	insecticide (LC_{50} = 1.23 µL/L air after fumigation of *O. basilicum* essential oil)	[94]
Ephestia kuehniella (major insect pest of dates both in field and in storage)	*O. basilicum* (linalool, methyl cinnamate, and eugenol)	insecticide (LC_{50} = 0.96 µL/L air after fumigation of *O. basilicum* essential oil)	[94]
Sitophilus zeamais (stored-grain pest)	*O basilicum* (linalool, estragole, α-humulene)	insecticide (LC_{50} = 0.014 mg/cm^3 air at 24 h after fumigation of *O. basilicum* essential oil)	[92]
Tribolium castaneum (stored-grain pest)	*O. basilicum* (linalool, estragole, α-humulene)	insecticide (LC_{50} = 0.02 mg/cm^3 air at 24 h after fumigation of *O. basilicum* essential oil)	[92]
Sitophilus oryzae (stored-grain pest)	*O. basilicum*	insecticide (30.7% mortality at 48 h after fumigation of *O. basilicum* essential oil) and repellent	[91]
Sitophilus orzyae (stored-grain pest)	*O. tenuiflorum* (eugenol and caryophyllene)	insecticide (LC_{50} = 963.3 µL/L air at 6 hours after essential oil exposure; inhibiting acetylcholinester)	[16]
Acanthoscelides obtectus (pest of beans)	*O. basilicum*	insecticide (74.94% mortality at 120 µL on day 15 after the oil application)	[17]
Aspergillus flavus (produce aflatoxins toxic)	*O. basilicum* (linalool, 1,8-cineol, eugenol)	antifungal agent (100% growth inhibition at 1000 µL/L essential oil)	[10]
Colletotrichum acutatum (anthracnose disease)	*Ocimum* sp. (methyl chavicol and linalool)	antifungal agent (MIC = 4 µL/mL)	[13]
Monilinia laxa (brown rot and grey mould rot of stone fruits)	*O. basilicum* (linalool, eugenol, estragole)	antifungal agent (control the growth of fungus on inoculated fruits)	[14]
Botrytis cinerea (brown rot and grey mould rot of stone fruits)	*O. basilicum* (linalool, eugenol, estragole)	antifungal agent (control the growth of fungus on inoculated fruits)	[14]
Penicillium italicum (rotting of citrus fruits)	*O. canum*	antifungal agent (enhance the shelf life of fungus inoculated oranges)	[15]
Cercospora purpurea (post-harvest pathogen of avocado)	*O. gratissimum*	antifungal agent (100% growth inhibited using ethanolic extract)	[98]
Aspergillus flavus (produce mycotoxins, aflatoxins toxic)	*O. basilicum*	antifungal agent (inhibits the production of aflatoxin B1)	[12]
Monilinia laxa (brown rot diseases of peach and nectarine)	*O. basilicum* var. purpurascens (estragole)	antifungal agent (inhibit the mycelium growth)	[99]

Table 4. *Cont.*

Pests	*Ocimum* Species (Volatile Active Compound)	Forms of Biological Activity	References
Monilinia laxa (brown rot diseases of peach and nectarine)	*O. tenuiflorum* (β-bisabolene and 1,8-cineole)	antifungal agent (inhibit the mycelium growth)	[99]
Aspergillus niger (associated with post-harvest rot of avocado pear)	*O. gratissimum*	antifungal agent (23.70% growth inhibition at 100% essential oil)	[11]
Aspergillus flavus (associated with post-harvest rot of avocado pear)	*O. gratissimum*	antifungal agent (51.93% growth inhibition at 100% essential oil)	[11]
Galactomyces candidum (associated with post-harvest rot of avocado pear)	*O. gratissimum*	antifungal agent (44.37% growth inhibition at 100% essential oil)	[11]
Trichoderma viride (associated with post-harvest rot of avocado pear)	*O. gratissimum*	antifungal agent (51.00% growth inhibition at 100% essential oil)	[11]
Lasiodiplodia pseudotheobromae (associated with post-harvest rot of avocado pear)	*O. gratissimum*	antifungal agent (66.74% growth inhibition at 100% essential oil)	[11]

5. Techniques for Enhancing the Essential Oil Efficiency

Encapsulation is a widely used process for generating an external membrane or coating material that protects or preserves sensitive bioactive, volatile, and quickly degradable substances from biochemical and thermal degradation [104]. Encapsulation is a technique that is commonly used in the flavour and fragrance industries to enhance both taste and scent. This technology also increases the efficacy of pesticides, fertilisers, and other toxic agrochemicals in agriculture, thereby improving productivity and food security. The active substances are encapsulated to regulate the release under accurate conditions (e.g., humidity, temperature, pH, and time) and to be active for a specific object (e.g., organisms or parts of the organisms). Moreover, encapsulation in agriculture can minimise harmful chemicals [105,106] and increase the efficiency of the natural extracts' action [66]. The encapsulation can be performed by coating with the material, creating core materials, filing in the internal phase or payload, and the substance's characteristics can be pure or mixed.

The coating materials are packing material, capsule, wall material, film, membrane, carrier, or outer shell [107]. They are usually made of natural or modified polysaccharides, gums, proteins, lipids, and synthetic polymers [108]. The organic flavour and the aroma of interest are low molecular weight compounds that are relatively volatile and very sensitive to open conditions (air, heat, light, and moisture). Depending on the applied encapsulation technique, the encapsulated essential oil products can be in powder, paste, or liquid forms [109,110]. Numerous techniques are available for encapsulating essential oils for agricultural uses, depending on the nature of the environment in which the products are applied.

5.1. Emulsification

To encapsulate the essential oil by the emulsion technique, the oil, including those of low polar molecules, has to be dissolved with emulsifiers such as gum Arabic and converted to droplets in water before further processes [111]. The droplets of basil oil are highly needed in food, perfumery, oral, and dental products. Emulsifiers such as proteins, phospholipids, and polysaccharides are used to maintain the stability of the essential oil emulsion. In addition, surfactants such as sugar esters and polyoxyethylene are also used to reduce the interfacial tension of the emulsion solution by electrostatic/steric

stabilisation [112]. This technique has been successfully proven to maintain the efficacy of the essential oil over harsh environmental conditions such as high temperature and provide the slow-release rate of the essential oil [113–115].

5.2. Complex Coacervation

Complex coacervation is an encapsulation method that links and forms two differently charged biopolymers in a solution with the appropriate pH value. The most commonly used biopolymers are gum Arabic, gelatine, carrageenan, chitosan, carboxymethyl cellulose, and pectin [116]. This technique is claimed to be suitable for application at high temperatures and humidity exposure [117,118].

5.3. Spray Drying

Spray drying is a method of forming a liquid essential oil into a powdery form. First, the essential oils are mixed in a solution containing wall materials such as maltodextrin, modified starch, gum, and the combination. Adding emulsifiers and homogenising agents is required to obtain smaller oil globules. Subsequently, the well-mixed solution is sprayed into hot air under high pressure, creating a mist that spreads in the drying chamber [119]. This results in a physical guard of the core matrix that protects the viability of essential oil during processing, storage, and transport [120].

5.4. Complexation

Encapsulation by complexation usually refers to the applied use of oligosaccharides such as cyclodextrins, specifically β-cyclodextrin, which are often used to encapsulate low polar substances such as essential oils. β-cyclodextrin, a cone-shaped molecule, comprises a network of compounds with 7 D-glucose α-1,4 glycosidic bonds. This structure allows essential oils to dissolve well in water and aids in fixing low-polarity substances and controlling evaporation [121]. The inclusion complex is said to increase the stability of the essential oils, particularly when exposed to sunlight [122].

5.5. Ionic Gelation

Essential oil encapsulation using the ionic gelation technique uses charged polymers with essential oils to form the solution. It is then moulded by dripping it into a crosslinking solution. Sodium alginate is a low-cost polymer often used to encapsulate essential oils due to its biocompatibility and biodegradability [123]. This alginate microsphere provides a protective structure from environmental factors such as volatilisation or oxidation. As for food, it facilitates the mobility of the essential oil into the animal digestive system [124].

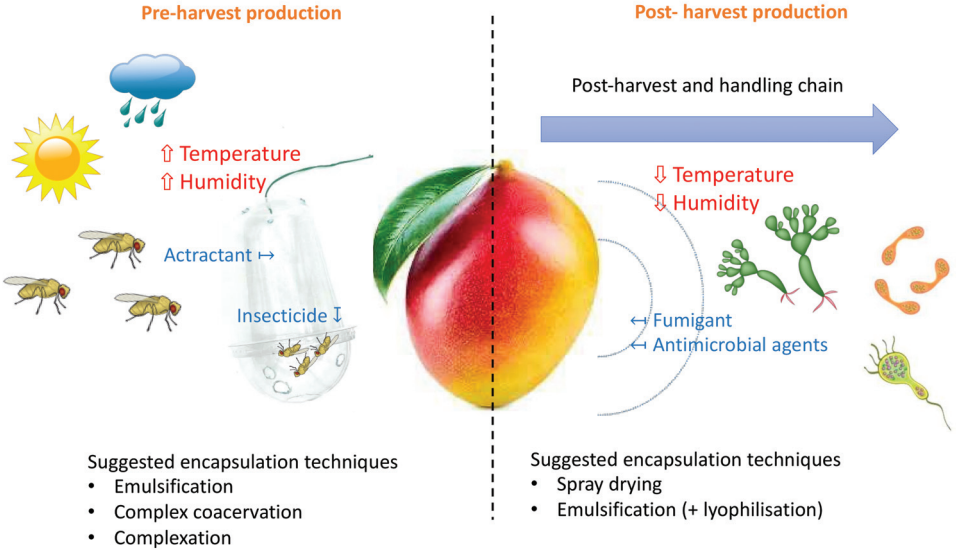
5.6. Nanoprecipitation

The process of hydrophobic component encapsulation using the nanoprecipitation (solvent displacement or interfacial deposition) technique involves first dissolving the essential oil in an organic solvent together with the polymers. The solution is then added to the water that is being stirred at the proper speed. The solution is then supersaturated, nucleated, and then it expands and coagulates [125]. This technique is suitable during post-harvest to increase the insecticidal efficiency against stored-grain insect pests [126]. There is, however, limited study on insect pest control during post-harvest and handling of tropical fruits.

Tangpao et al. [66] studied the Oriental fruit fly-attracting ability of methyl eugenol plant-based essential oils and encapsulated the oil using an adapted complexation with the paste method of different wall materials. The holy basil essential oil was found to have the ability to attract the Oriental fruit flies, and encapsulation with maltodextrin and gum Arabic at a ratio of 75:25 could enhance its effectiveness in attracting the flies in mango orchard. For this purpose, the paste method is the economical and straightforward technique to encapsulate the essential oil using the chemical and mechanical reaction between the core and wall materials [127]. In addition, the encapsulation of basil essential

oil by freeze-drying technique is encouraged to prevent the loss of such heat-sensitive active volatiles that are unstable in aqueous solution [128].

The antimicrobial properties of essential oils have led to research interest in their applications during the post-harvest and storage of fruits. However, the downside is that the strong scents from essential oils could interfere with the true aroma of the produce. This is generally mitigated by the nanoemulsion technique with sodium alginate or pectin-based edible coating and the high-pressure homogenisation technique [112]. All in all, several considerations should be taken into account when applying the essential oil in agricultural productions, such as its volatilisation nature [129] and the activity losses due to the exposure to ultraviolet light, temperature, humidity, and oxygen [130]. More importantly, the release-control rate of the products needs to be investigated [131,132]. Scheme 1 illustrates the possible encapsulation approaches to increase the efficiency of the essential oil during tropical fruit production.



Scheme 1. Enhancing the efficiency of basil volatile organic compounds during pre- and post-production of the tropical fruits.

6. Conclusions

This review provides essential information for understanding the usefulness of volatile organic compounds from diverse types of basil essential oils and their biological activities. Moreover, as far as sustainable food production in the tropical region is concerned, it is interesting to value-add the natural products from the commonly available resources. Several studies validated the bioassays of these beneficial components during the pre- and post-harvest stages of food crop development. However, the limitation is that essential oils generally decompose fast when exposed to the environment. Consequently, encapsulation techniques are recommended to improve its stability and control its release rate. The option of choice depends on the targeted applications and better-controlled release performance of the essential oils.

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Review

Opuntia genus in Human Health: A Comprehensive Summary on Its Pharmacological, Therapeutic and Preventive Properties. Part 1

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Abstract: *Opuntia* spp. is a diverse and widely distributed genus in Africa, Asia, Australia, and America. Mexico has the largest number of wild species; mainly *O. streptacantha, O. hyptiacantha, O. albicarpa, O. megacantha and O. ficus-indica.* The latter being the most cultivated and domesticated species. Throughout history, plants and their phytochemicals have played an important role in health care and *Opuntia* spp. has shown a high nutritional, medicinal, pharmaceutical, and economic impacts. Its main bioactive compounds include pigments (carotenoids, betalains and betacyanins), vitamins, flavonoids (isorhamnetin, kaempferol, quercetin) and phenolic compounds. Together, they favor the different plant parts and are considered phytochemically important and associated with control, progression and prevention of some chronic and infectious diseases. This first review (Part 1), compiles information from published research (in vitro, in vivo, and clinical studies) on its preventive effects against atherosclerotic cardiovascular diseases, diabetes and obesity, hepatoprotection, effects on human infertility and chemopreventive and/or antigenotoxic capacity. The aim is to provide scientific evidences of its beneficial properties and to encourage health professionals and researchers to expand studies on the pharmacological and therapeutic effects of *Opuntia* spp.

Keywords: *Opuntia* spp.; phytochemicals; biological activities; antioxidant capacity; chemopreventive effect

1. Introduction

The Traditional Medicine/Complementary and Alternative Medicine (TCAM) concept includes any practice, knowledge and belief in health that incorporates medicine based on plants, animals and/or minerals, spiritual therapies, manual techniques and exercises applied individually or in combination to improve human health. The World Health Organization (WHO) considers that TCAM have shown favorable factors that contribute to an increasing acceptance worldwide, such as easy access, diversity, relatively low cost and, most importantly, relatively low adverse toxic effects in comparison with allopathic

medicine where these effects are frequently attributed to synthetic drugs. For this reason, TCAM continues to be used by different populations to treat and/or prevent the onset and progression of chronic diseases including obesity, diabetes, hypertension, atherosclerosis, and cancer [1–3].

Throughout human history, plants and their phytochemicals have played an important role at improving human health care. *Opuntia* spp. species have specifically shown many beneficial properties and high biotechnological capacity. These plants classified as angiosperm dicotyledonous are the most abundant of the Cactaceae family and are importantly distributed in America, Africa, Asia, Australia, and in the central Mediterranean area. Due to their capacity to store water in one or more of their organs, they are considered "succulent plants" whose cultivation is ideal in arid areas since they are very efficient to generate biomass in water scarcity conditions [4–7].

Most opuntioid cacti have flat and edible stems called cladodes (CLD), paddles, nopales or stalks. Generally, young CLDs (also called nopalitos) are eaten as a vegetable in salads, while their fruits [called cactus pear fruits (tunas) or prickly pear fruits (PPFs)] are widely eaten as fresh seasonal fruit. Prickly pears are oval berries with lots of seeds throughout all the pulp and a semi-hard bark that contains thorns. They are grouped in different colors (red, purple, orange/yellow, and white). Generally, the fruit with white flesh and green skin is the most consumed as food [4–7]. Some evidences indicate that *Opuntia* plants have been consumed by humans for more than 8000 years and due to their easy adaptation and spread in different types of soil, their domestication process in man-made environments has increased favoring the constant collection of CLD and PPFs [7–10].

2. *Opuntia* genus in Mexico

Opuntia spp. is a diverse and widely distributed genus in the American Continent. However, Mexico has the largest number of wild species. The most representaive are *O. streptacantha* (OS), *O. hyptiacantha* (OH), *O. albicarpa* (OA), *O. megacantha* (OM) and *O. ficus-indica*. The latter is highly cultivated and domesticated species due to its nutritional, medicinal, pharmaceutical, and economic impacts. It is believed to be a secondary crop with fewer thorns derived from OM, (a native species from central Mexico) [4,7,8,10,11]. Currently, *O. ficus-indica* (OFI) has become as important a vegetable crop as corn and agave-*tequila*; its economic relevance is significantly increasing in our country and in other parts of the world, especially for improving health when nopal and prickly pear are included in a diet. Therefore, the OFI domestication process has favored changes in the texture, flavor, size, color, quantity and quality of the cladodes and their fruits [4,7,8]. Mexico and Italy are the main producing and consuming countries of the approximately 590,000 ha cultivated around the world. The Annual Mexican production can reach 350,000 tons; for this reason, our country represents approximately 90% of the total production worldwide. In addition, Mexico is the main producer of prickly pear, representing more than 45% of world production; however, only 1.5% of this production is exported [4,7,11,12].

3. Nutritional Value, Bioactive Compounds and Main Mechanisms of Action Involved

Various investigations where different extraction methods were used have documented the nutritional value of *Opuntia* spp. Most of these studies coincide in the differences among the phytochemical composition of their plant parts (fruits, roots, cladodes, flowers, seeds and stems) and the wild and domesticated species. These can be attributed to environmental conditions (climate, humidity), the type of soil that prevails in the cultivation sites, the age of maturity of the cladodes, and the harvest season [5–7]. In general, opuntioid cacti contain a large amount of water (80 and 95%), carbohydrates (3–7%), proteins (0.5–1%), soluble fiber (1–2%), fatty acids (palmitic, stearic, oleic, vaccenic and linoleic) and minerals [Potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), chrome (Cr) and sodium (Na)]. They also have viscous and/or mucilaginous materials [made up of D-glucose, D-galactose, L-arabinose, D-xylose and polymers such as β-D-galacturonic acid linked to (1–4)

and L-rhamnose residues linked with R (1–2)] whose function is to absorb and regulate the amount of cellular water in dry seasons [5–7,13]. Among the main bioactive compounds of prickly pear highlight the pigments (carotenoids, betalains, betaxanthins and betacyanins), vitamins (B1, B6, E, A, and C), flavonoids (isorhamnetin, kaempferol, quercetin, nicotiflorine, dihydroquercetin, penduletin, lutein). Rutin, aromadendrine, myricetin vitexin, flavonones and flavanonols) and phenolic compounds (ferulic acid, feruloyl-sucrose and synapoyl-diglycoside) [5–7,13–16].

Specifically, the CLDs and prickly pears of OFI have shown several kinds of bioactive compounds, among which flavonoids (such as quercetin, kaempferol, isorhamnetin), essential amino acids [Glutamine (Glu), arginine (Arg), leucine (Leu), isoleucine (Ile), lysine (Lys), valine (Val), and phenylalanine (Phe)], vitamins (B1, B6, E, A, and C), minerals (mainly K and Ca), and betalains [such as betaxanthins (betanin and indicaxanthin) and betacyanins (betanidin, isobetanin, isobetanidine, and neobetanin) (Table 1) [5–7,13–16].

Table 1. Main bioactive compounds in different anatomical parts of *O. ficus-indica*.

Chemical Group	Bioactive Compound
	Cladodes (CLD)
Flavonoids	Quercetin (2.0–40 mg/100 g), isoquercetin (2.29–39.67 mg/100 g), isorhamnetin-3-O-glucoside (4.59–32.21 mg/100 g), kaempferol (2.2 g/kg), nicotiflorin (2.89–146.5 mg/100 g), rutin (2.36–26.17 mg/100 g)
Phenoliccompounds	Gallic acid (0.6–2 mg/100 g), coumaric (14–16 mg/100 g), 3,4-dihydroxybenzoic (0.06–5.0 mg/100 g), 4-hydroxybenzoic, ferulic acid (0.5–34 mg/100 g)
Amino acids	Glu (36 g/100 g), Arg (5.0 g/100 g), Leu (2.7 g/100 g), Ile (3.97 g/100 g), Lys (5.2 g/100 g), Val (7.7 g/100 g), Phe (3.5 g/100 g), Glu (36.1 g/100 g
Minerals	K and Ca (mainly calcium oxalate crystals). Amounts ranging from 230 to 5500 mg/100 g
Vitamins	E (2182 mg/100 g), A (7–10 mg/100 g), C (7–22 mg/100 g), B1 (0.14 mg/100 g), B2 (0.60 mg/100 g), B3 (0.46 mg/100 g)
	Prickly pear fruits (tunas)
Flavonoids	Kaempferol(53.2 mg/100 g), quercetin(90 µg/g), isorhamnetin(49.4 µg/g)
Amino acids	Lys (0.63 g/100 g), Met (2.0 g/100 g), Glu (12.5 g/100 g), Taurine (15.7 g/100 g)
Minerals	K (161 mg/100 g), Ca (27 mg/100 g), Mg (27 mg/100 g)
Vitamins	E (527 mg/100 g), A (5 mg/100 g), C (34–40 mg/100 g)
Organic acids	Maleic, malonic, succinic, tartaric, and oxalic. Total average of 0.36 to 8.50 mg/g
Betalains	Betaxanthins (25.4 mg indicaxanthins/100 g), Betacyanins (15.2 mg betanin/100 g), Betalains (40.6 mg/100 g)
	Seeds
Phenolic compounds	Ferulic acid (3–17 mg/100 g), sinapoyl-diglucoside (13–22 mg/100 g), synapoyl-glucose, feruloyl-sucrose (8–17 mg/100 g). Total average of compounds 48–89 mg/100 g
Minerals	Mainly K and P (160–150 mg/100 g). Lower proportions of Mg (70 mg/100 g), Na (66 mg/100 g) and Ca (16 mg/100 g)
Sterols	β-sitosterol (67.0–21.0 g/kg) and campesterol (1.6–8.7 g/kg)
Fatty acids	Palmitic acid (9–20 g/100 g), oleic acid (16–18 g/100 g), linoleic acid (53–70 g/100 g)
	Pulp and peel
Flavonoids	Quercetin (4–9 mg/100 g), isorhamnetin (3–90 mg/100 g), kaempferol (0.2–0.8 mg/100 g), luteolin (0.8–1.0 mg/100 g), isorhamnetin glycosides (50–60 mg/100 g)
Phenolic compounds	Ferulic acid, sinapoyl-diglucoside, feruloyl-sucrose isomer. Total average of compounds 218.8 mg/100 g
Minerals	More K (161 mg/100 g) than Ca and Mg
Sterols	β-sitosterol (67.0–21.0 g/kg) and campesterol (1.6–8.7 g/kg)
Fatty acids	Palmitic acid (34 g/100 g), oleic acid (10.8 g/100 g), linoleic acid (37 g/100 g), linolenic acid (12.6 g/100 g)

Table 1. *Cont.*

Chemical Group	Bioactive Compound
	Flowers
Flavonoids	Kaempferol (300–400 mg/100 g), quercetin (400–700 mg/100 g), isorhamnetin glycosides [isorhamnetin 3-O-robinobioside (4269 mg/100 g), isorhamnetin 3-O-galactoside (979 mg/100 g), isorhamnetin 3-O-glucoside (724 mg/100 g)]
Organic acids	Mainly gallic acid (1600–4900 mg/100 g)

Table modified from El-Mostafa et al. (2014) [6]. Average and most significant data obtained from Angulo-Bejarano et al. (2014) and El-Mostafa et al. (2014) [5,6]. Potassium (K), Phosphorus (P), Magnesium (Mg), Sodium (Na), Calcium (Ca), methionine (Met), glutamine (Glu), arginine (Arg), leucine (Leu), isoleucine (Ile), lysine (Lys), valine (Val), and phenylalanine (Phe).

Various studies have shown the action of phytochemicals as substrates to activate different biochemical reactions that provide important health benefits. For that reason, they could be included in the definition of nutraceutical: "Any non-toxic food extract supplement that has been scientifically proven to be beneficial to health both intreating and preventing diseases" [5,17].

In this context, opuntioid cacti reveal different mechanisms of action that can be interrelated and favor their biological effects. In general, they are organized in 7 groups: (I) Inhibition of the absorption of substances, favoring the absorption of protective agents and/or modification of the intestinal flora (action of soluble fiber and ascorbic acid), (II) Scavenging of reactive oxygen species and/or protection of DNA nucleophilic sites (antioxidant action), (III) Anti-inflammatory activity, (IV) Modification of transmembrane transport (effect of short-chain fatty acids and calcium in the diet), (V) Modulation of xenobiotic metabolising enzymes, inhibition of mutagen agents activation and induction of detoxification pathways (flavonoids, polyphenols and índoles), (VI) Enhancement of apoptosis (action of some flavonoids), and (VII) Maintenance of genomic stability (effect of some vitamins, minerals and polyphenols) [3,5–7,14–17].

Due to the bioactive compounds of *Opuntia* spp. the different plant parts can be associated to control, progression, and prevention of chronic and infectious diseases. However, it is relevant to comment that the Cactaceae family contains approximately 130 genera and 1500 species, which favors a wide genetic diversity that in conjunction with environmental conditions (climate, humidity), soil type, age of maturity of the cladodes and the harvest season generates differences in the phytochemical composition between wild and domesticated specie, inducing changes in their nutritional values and/or functional properties. This first review (Part 1), focuses on information from published research (in vitro, in vivo and clinical studies) on its action in atherosclerotic cardiovascular diseases, diabetes and obesity, hepatoprotection, effects on human infertility and chemopreventive and/or antigenotoxic capacity; which will be discussed below.

4. Pharmacological, Therapeutic and Preventive Properties

4.1. Effects on Atherosclerotic Cardiovascular Diseases

The term atherosclerotic cardiovascular diseases (ASCVD) encompasses conditions that affect the blood vessels and the heart as a consequence of the thickening and hardening of medium and large-caliber arteries. ASCVD involves diseases of the cardiovascular system that share similar characteristics regarding their cause, pathophysiology, prognosis, and treatment. They are one of the main causes of mortality in the world (including Mexico) and unfortunately, their incidence is increasing [18].

In general, atherosclerosis is an inflammatory and chronic process characterized by the progressive occlusion of arteries where there is retention, oxidation, and modification of lipids in the form of fatty stretch marks, whose development can generate endothelial dysfunction, inflammation, and thrombosis. When serum concentrations of LDL-cholesterol (LDL-Cho) rises significantly, it penetrates the arterial walls to accumulate among the cells where reactive oxygen species (ROS) are induced and produce oxidation of LDL

(Low-density lipoprotein) that generates the release of pro-inflammatory cells (such as monocytes and neutrophils).

Specifically, monocytes become macrophages, which promote the progression of the lesion by stimulating an inflammatory cascade. Various situations that cause endothelial damage have been identified, such as hypercholesterolemia, hypertension, diabetes, obesity, and smoking [7,18,19], which together with genetic predisposition are considered traditional risk factors; Several of these factors are related to changes in lifestyle, which has contributed to generating certain strategies (especially those focused on smoking and poor nutritional habits) that reduce the possibility of cardiovascular risk. In this context, both CLD and PPFs from *Opuntia* spp. have shown antiatherogenic, antihyperlipidemic, and antihypercholesterolemic properties due to their soluble fiber content, flavonoids, phenolic compounds and fatty acids. In the case of fiber, composed of substances (such as cellulose, hemicelluloses, pectin, lignin and gums) resistant to digestive enzymes, the hypolipidemic effect is attributed to them due to their binding to dietary fat which promotes its excretion by fecal route with the consequent reduction of body fat [7,14,20,21].

On the other hand, some flavonoids, phenolic compounds and fatty acids, are considered phytochemicals with the same activity and anti-inflammatory effects. Such properties are supported by its antioxidant capacity. Specifically, quercetin 3-methyl ether, obtained from cladode extracts of OFI, has shown potential to lower cholesterol (Cho); while omega-6 linoleic acid from cactus seed oil is considered a precursor of arachidonic acid with a hypocholesterolemic effect [6,7]. Also, betalains [such as betanin and indicaxanthin (Ind)] from PPFs have evidenced to protect the vascular endothelium from inflammation and cytokine-induced oxidative alteration [such as tumor necrosis factor alpha (TNF-α)] by inhibiting intercellular adhesion molecule-1 (ICAM-1) [22].

Table 2 shows the most relevant studies of the hypolipidemic, hypocholesterolemic and antiatherogenic properties of *Opuntia* spp. In summary, from 1996 to date, 4 out of 17 have been in vitro studies; 7, using laboratory animals (mainly rodents); 5, developed with patients (clinical studies) and only one where a systematic review was made. Garcia-Diez et al. (1996) were the first researchers to explore a diet supplemented with pectin (extracted from nopal) in the metabolism of Cho and bile acids of Wistar rats. After four weeks, they obtained lower concentrations of Cho in serum and liver; as well as a higher activity of the regulatory enzymes of Cho [3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) and cholesterol 7 alpha-hydroxylase (Cho7AH) [23]. Possibly, these results prompted other researchers to analyze the intake of PPFs (in pulp form) in patients suffering from isolated heterozygous familial hypercholesterolaemia [24] and primary hypercholesterolaemia [25]; as well as to extract a glycoprotein (GOFI) from the nopal to be orally administered to mice for two weeks and to show that in both cases the plasma levels of triglycerides (TG), tCho and LDL were reduced [26]. In 2012, the lethal dose 50 (LD_{50}) of a methanolic extract of *O. joconostle* (OJ) seeds was found to be greater than 5000 mg/kg and that mice fed with a hypercholesterolemic diet along with this oral extract could reduce the concentrations of TG, tCho and LDL-Cho [27]. A similar phenomenon took place when evaluating a food supplement (*NeOpuntia*) obtained from dehydrated leaves of OFI on blood lipid parameters of 59 women after 6 weeks of treatment [28].

Regarding the antiatherogenic potential, during 2015 this property was tested by using different powdered cladodes of *Opuntia* (OS, OH, OA, OM, OFI) against the oxidation of LDL caused by vascular endothelial cells and the toxicity of 4- hydroxynonenal under normal conditions (Apc +/+) and in immortalized preneoplastic epithelial colon cells (Apc min/+). The conclusion was that all powders significantly inhibited the oxidation induced by incubation with murine endothelial cells and the formation of foam cells of murine macrophages RAW 264.7 [29].

Table 2. Studies testing for hypolipidemic and antiatherogenic effects of *Opuntia* spp.

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
	Hypolipidemic and hypocholesterolemic effect		
In vivo	The objective was to evaluate the effect of pectin (extracted from CLD) in the metabolism of Cho and bile acids in male Wistar rats. The animals were fed during 4 weeks with a pectin supplemented diet (7 g/100 g) where Cho regulatory enzymes (HMG-CoA reductase and Cho7AH), concentrations of circulating and hepatic lipids and the excretion of fecal bile acids were measured.	After this period, lower concentrations of Cho were found in serum and liver, as well as a significant excretion of fecal bile acids and a greater activity of Cho7AH and HMG-CoA reductase. It was concluded that pectin favors the decrease of hepatic cholesterol and its serum concentrations.	[23]
Clinical study	Considering that the PPFs is a traditional food of the American indigenous population, the effect of its pulp was evaluated in 15 patients of both genders who suffered from isolated heterozygous familial hypercholesterolemia (FH).	After a daily consumption of 250 g of pulp for four weeks, tCho and LDL-Cho had diminished in all patients, being more significant in men. Likewise, when analyzing the oxidative damage using the biomarker 8-epi-PGF (2 alpha), a decrease in plasma, serum, and urine was evidenced. The results suggest that the consumption of PPFs can reduce oxidative lesions and benefit the cardiovascular system.	[24]
Clinical study	In this pilot study the aim was to evaluate the effects of PPFs on lipid metabolism. A group of 24 men with FH (without diabetes and obesity) consumed its pulp (250 g/day) for eight weeks.	After this period, a significant reduction of tCho (12%), LDL-Cho (15%), TG (12%) and apolipoprotein B (9%) was observed. The conclusion was that PPFs may show a hypocholesterolemic action due to the content of soluble fiber (such as pectin).	[25]
In vivo	A glycoprotein (GOFI) was isolated from *O. ficus-indica var. Saboten* to determine its ability to reduce the plasma lipid level through scavenging of intracellular radicals in Triton WR-1339-induced mice. GOFI was orally administered to the animals (50 mg/kg) for two weeks.	The results showed that GOFI reduced the plasma levels of triglycerides (TG), total concentration of cholesterol (tCho) and Low-density lipoproteins (LDL) induced by Triton WR-1339. In addition, a decrease in the level of thiobarbituric acid-reactive substances (TBARS) and an increase in the enzymatic activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were observed. The hypolipidemic effect is probably related to the antioxidant capacity of GOFI.	[26]
In vivo	The purpose of this study was to evaluate the hypolipidemic effect of a methanol extract (MeOH) from *O. joconostle* (OJ) seeds in mice fed with a hypercholesterolemic diet.	It was initially found that the oral lethal dose 50 (LD_{50}) was greater than 5000 mg/kg. The supplementation of the extract (doses of 1, 2 and 5 g/kg) significantly decreased the concentrations of TG, tCho and LDL-cholesterol (LDL-Cho). The hypolipidemic effect of MeOH was probably due to the phenolic composition of the seeds and the dose administered.	[27]
Clinical study	Considering that NeOpuntia is a nutritional supplement obtained from dehydrated leaves of OFI, the purpose of this monocentric study, randomized, and placebo-controlled was to analyze its effect on blood lipid parameters of 59 women. During 6 weeks, the individuals consumed balanced diets with controlled lipid intakes plus NeOpuntia capsules (1.6 g dose per meal) and TG, LDL-Cho, and HDL-Cho (High-density lipoprotein cholesterol) levels were measured.	Most of the women showed an increase in HDL-Cho levels and a decrease in blood levels of TG and LDL-Cho. The results suggest that NeOpuntia may reduce cardiovascular risks.	[28]

Table 2. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
Clinical study	After subjecting 10 patients with FH to a dietary treatment with PPFs for 6 weeks, the hypolipidemic potential of this fruit was evaluated by the uptake of autologous (123) I-radiolabeled LDL.	The results showed a relevant increase in the hepatic uptake of LDL and consequently, lower levels of tCho and LDL-Cho in the circulating blood. These findings suggest that the beneficial effect is related to a positive regulation of the receptor [(123) I-LDL].	[30]
In vivo	Stem intake of *O. humifusa* (OHF) was examined on the regulation of lipid concentrations in Sprague-Dawley rats with streptozotocin (STZ) injection-induced diabetes mellitus (DM). The animals were treated orally with two doses of OHF (150 and 250 mg/kg per day) for seven weeks.	Both treatments favored a level lowering of TG, tCho and LDL-Cho. Furthermore, the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were significantly reduced compared to the DM control group. These results suggest that OHF is potentially hypolipidemic.	[31]
In vivo	The capacity of ODP-Ia (main component of *O. dillenii Haw* (OdHw) polysaccharides) on lipid concentration in hyperlipidemic rats induced by high-fat emulsion was analyzed.	After the oral administration of ODP-Ia, serum lipid levels and liver concentrations of tCho and TG significantly decreased. The same treatment increased the activity of cholesterol acyltransferase and SOD (serum and hepatic) and inhibited the action of HMG-CoA reductase and the content of malondialdehyde (hepatic and serum). In addition, by means of a histopathological analysis, the inhibition of the infiltration of inflammatory cells was observed. Together, these results suggest that ODP-Ia is a natural product that can be used in the treatment of hyperlipidemic diseases and that their mechanisms of action are related to the antioxidant potential and the modulation of the enzymes involved in the metabolism of Cho.	[32]
In vitro	In this test, the effect of piscidic acid and some derivatives of isorhamnetin (Isorhamnetin glucosyl-rhamnosyl-rhamnoside, isorhamnetin-glucosyl-rhamnosyl-pentoside, isorhamnetin-3-O-glucosyl-pentoside, Isorhamnetin-3-O-rutinoside) was evaluated on the absorption of Cho in a monolayer of Caco-2 cells.	The results indicated an approximate 38% reduction in Cho permeation, while for phenolic compounds it was 6% (isorhamnetin) and 9% (piscidic acid). It was also observed that the mixture of both phytochemicals showed an IC50 of 20.3 µg/mL (inhibition of the HMG-CoA enzyme), while for the piscidic acid it was 149.6 µg/mL. This value was slightly exceeded by isorhamnetin derivatives. The data suggest considering OFI as a promising plant for the development of new pharmaceuticals with hypocholesterolemic potential because its bioactive compounds could bind to the active site of the HMG-CoA enzyme.	[33]
Systematic review	Despite the concise benefits of *Opuntia* spp. in ASCVD, there is still some confusion about the lipid-lowering effect between its CLD and PPFs. Due to that confusion a systematic review of the characteristic was carried out (from February to September 2019) in the main electronic databases, considering both plant parts and using keywords such as tCho, LDL-Cho, HDL-Cho and TG. Eleven articles (6 from PPFs, 4 from CLD and 1 from commercial products) met the established criteria.	In summary, the consumption of PPFs is associated with significant reductions in tCho, LDL-Cho and TG; while in CLD the lipid-lowering effect is less and there is a datum on a significant increase in HDL-Cho. Possibly, the discrepancies in this effect are caused by the different chemical compositions between CLD and PPFs. Therefore, it would be more feasible to identify the components of *Opuntia* spp. with greater precision in future studies.	[34]

Table 2. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
In vivo	The content of the total phenolic content and the antioxidant and antihyperlipidemic activities of the seed oil of *O. dillenii Haw* (OdHw) were evaluated. Using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and the Folin-Ciocalteu method, the antioxidant activity and phenolic content were tested. The other property was evaluated in albino mice fed a high-fat diet plus OdHw (2 mL/kg).	The oil showed a high phenolic content and DPPH scavenging activity. It also presented a significant antihyperlipidemic effect by improving the lipid profile of the animals; which suggests that this property is related to the antioxidant activity and the phenolic content of the plant's seeds.	[35]
		Antiatherogenic effect	
In vitro	As is known, atherosclerosis is a chronic process where macrophages stimulate inflammatory cascades that promote endothelial dysfunction and allow the constitutive form of ICAM-1 to be expressed. Therefore, the ability of two betalains (betanin and Ind) from PPFs to protect the endothelium from cytokine-induced oxidative alteration by inhibiting ICAM-1 was evaluated. Human umbilical vein endothelial cells (HUVECs) were stimulated with TNF-α and flow cytometry measurements were subsequently performed by incubation with anti-human-ICAM-1.	The results showed that both pigments were able to slightly inhibit ICAM-1 expression up to a micromolar concentration. The antioxidant evaluation of these phytochemicals opens the possibility to develop pharmacological studies that are related to other pathologies characterized by endothelial dysfunction such as atherothrombosis, low limb ischemia, and stroke.	[22]
In vitro	The purpose of the study was to investigate the protection of different *Opuntia* CLDs (OS, OH, OA, OM, OFI) in powder form against LDL oxidation caused by vascular endothelial cells and the toxicity of 4-hydroxynonenal under normal conditions (Apc +/+) and in preneoplastic immortalized epithelial colon cells (Apc min/+).	All powders showed a significant inhibition of the oxidation induced by incubation with murine endothelial cells and the foam cell formation of RAW 264.7 murine macrophages. Furthermore, they reduced murine endothelial cell cytotoxicity and colon cancer development in the in vitro model. The conclusion was that the therapeutic potential of cladodes is related to their antioxidant capacity and their content of phenolic acid and flavonoids.	[29]
In vitro	Since macrophage apoptosis induced by 7-ketocholesterol (7-KC) is a key event in the development of human atheromas, the study of the effect of Ind on 7-KC-induced apoptosis of human monocyte/macrophage THP-1 cells was considered. The proapoptotic potential of 7-KC was evaluated by cell cycle arrest, phosphatidylserine exposure in the plasma membrane, variation of nuclear morphology, and activation of the antagonist Bcl-2 (B-cell lymphoma 2) of cell death.	During the first 24 h, elevated ROS levels were observed, preceding the overexpression of NADPH oxidase-4 (NOX-4) and the elevation of cytosolic Ca^{2+}; confirming the 7-KC-dependent activation of the redox-sensitive NF-κB; while the co-incubation of Ind (2.5 μm) prevented such pro-apoptotic events. This pigment of PPFs might protect against atherogenic toxicity of 7-KC by inhibiting overexpression of NOX-4, inhibiting the activation of Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF -κB), the maintenance of cellular redox balance and Ca^{2+} homeostasis.	[36]
In vivo	The purpose of the study was to determine whether CLDs of OS and OFI could prevent the development of atherosclerosis in ApoE(-)KO mice. Likewise, using both *Opuntia* species, the concentration of ROS, the kinetics of LDL oxidation by murine CRL2181 endothelial cells, and the capacity of the inflammatory process to induce the adhesion of monocytes in the activated endothelium and the formation of foam cells were determined.	The evidence showed that OS and OFI had significantly reduced the extracellular generation of superoxide anion, the oxidation of LDL and its subsequent signaling cascade (including the expression of ICAM-1 and NFκB). A reduction in atherosclerotic lesions and 4-hy-droxynonenal adducts in the vascular wall of mice was also observed. Therefore, it is suggested that both *Opuntia* species (wild and domesticated) show antioxidant, anti-inflammatory, and anti-atherogenic properties.	[37]

Table 2. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
Clinical study	Considering that the antioxidant properties of OFI have been associated with a reduction in body fat, the effects of a dietary supplementation of a 3% OFI extract (500 g per week) was analyzed in 49 individuals (13 men and 36 women). The complete profile of the LDL subclass was evaluated by gel electrophoresis for one month.	The study showed in a percentage increase of LDL-1 and a concomitant reduction in LDL-2; which suggest that OFI extract may have beneficial effects on LDL particle size making them less atherogenic.	[38]

4.2. Effects on Diabetes and Obesity

The metabolic syndrome is the set of carbohydrate and lipid metabolic abnormalities that describes the increasing incidence of type 2 diabetes mellitus (DM2) associated with abdominal obesity (AO), insulin resistance, and cardiovascular disease [14,39]. DM2 is a multifactorial disease that includes genetic determinants of individual susceptibility and environmental factors of lifestyle. It is considered a serious public health problem, being among the leading causes of death worldwide.

The high social costs and implications for hospital systems are some of the relevant consequences. The worldwide increase of the metabolic syndrome is estimated to 360 million people by 2030. It is characterized by a decrease in glucose uptake stimulated by insulin (insulin resistance) and once the disease is established and there is poor control in the patients, macrovascular complications may develop (including atherosclerosis) as well as microvascular abnormalities (such as retinopathy, nephropathy, and neuropathy). Furthermore, in the long term, grave problems may result in the kidney and the heart [5,7,14,39].

On the other hand, obesity (also considered a public health problem) has significantly increased as a result of rapid urbanization, growing technology, altered eating habits, and decreasing physical activity. Specifically, Mexico faces a challenging situation due to its high incidence. The statistical data indicate that in adults it occurs in 33% (higher prevalence in women) and approximately 15% in children [7].

There is a close relationship between AO and insulin resistance. This is due to the fact that AO implies an increase in fat at the visceral level (mainly in the liver, muscles and pancreas), which induces the formation of adipokines in the fat tissue and favors pro-inflammatory and prothrombotic states, which contribute to the development of insulin resistance, hyperinsulinemia and endothelial dysfunction (cardiovascular disease) [5,39].

Adiponectin, in particular, decreased; which is a situation associated with an increase in TG, a decrease in HDL, an elevation of apoliprotein B, and the presence of dense LDL particles, contributing to the atherothrombotic state that represents the inflammatory profile of visceral adiposity [39]. It is also known that oxidative stress (OXs) is related to insulin resistance and obesity and that a high concentration of ROS can induce and/or favor the development of both diseases.

An interesting observation is that low levels of adiponectin are usually the result of this high concentration; also, the production of ROS in adipocytes is associated with insulin resistance and alterations in serum levels of adiponectin with the consequent inflammatory response [5,40].

The herbal treatments and traditional plant-based medicines are increasingly popular due to their low-cost with apparently fewer side effects for treating DM2 and AO. In general, four possible mechanisms of action have been directed at *Opuntia* spp.

The first is related to its content of fiber, pectin and mucilage that slow down the speed of digestion and/or intestinal absorption of glucose and fatty acids [5,7,25,34,41,42]. The second focuses on improving the postprandial glucose response and stimulating insulin secretion through a direct action on pancreatic β cells after a dietary intake that includes nopal [43–45].

Another hypothesis lies on its antioxidant properties. As it is mentioned above, OXs plays a fundamental role in the development of atherosclerosis and cardiovascular diseases, the main complications of DM2 and AO [40]. A relevant data on this mechanism is its content of polysaccharides (arabinose, xylose, fructose, glucose, galacturonic acid and rhamnose) that have demonstrated anti-inflammatory activity and ability to isolate ROS. This supports the fact that these polysaccharides can reduce hepatic lipoperoxidation, maintaining the tissue function and improving the target cells response to insulin [46,47]. The final proposal is that Cr (III), present in the CLD of nopal and extracts of the pulp of PPFs, is an important element in mammals to maintain the balance of carbohydrates and lipids. Some studies have validated this property, finding a positive effect of Cr (III) on insulin signaling and/or function; helping to improve its systemic sensitization and reduce plasma glucose under fasting conditions [48–50].

After the studies related to cardiovascular diseases, the greatest scientific interest in *Opuntia* spp. has focused on its ability to treat DM2 and AO. Approximately 47 scientific articles have been published since the 1980s; 10 of which belong to clinical investigations, 4 are in vitro studies, 24 in vivo tests (using rodents, rabbits and pigs), and 9 of them are systemic reviews.

Initially, the research of Ibañez and Meckes (1983) stands out, who evaluated the hypoglycemic effect of a semi-purified fraction of OS in rabbits and observed that the powder obtained would produce an effect similar to the traditional extract of OS stems [51]. Years later, with the same animal model, another study confirmed that OS decreased the area under the glucose tolerance curve and the hyperglycemic peak [52].

Likewise, they observed that the red juice obtained from *O. dillenii Haw* (OdHw) increased plasma insulin levels in normoglycemic and alloxane (Allox) -induced diabetic rabbits [53]. Another frequently used experimental model is the administration of streptozocin (STZ) to induce diabetes in rodents. A dose of 1.0 mg/kg/day of a purified extract of *O. fuliginosa* (Of) decreased blood glucose levels and glycosylated hemoglobin (HbA1c) in diabetic rats [54].

Not only the species Of has demonstrated this property, since the juice of OdHw, a rich source of fiber, minerals and vitamins, has also reduced glucose levels [55]. On the other hand, four extracts of *O. Milpa Alta* [Aqueous, petroleum ether, ethyl acetate (EtOAc), and butanol (BuOH)] were tested in STZ-induced diabetic mice and they also managed to lower glucose levels [56].

Hahm et al. (2011) studied the intake of three doses of OHF (150, 250 and 500 mg/kg/day) for 7 weeks on the regulation of blood glucose in diabetic rats; their conclusion was that all doses reduced this blood parameter to values comparable to the DM control group. In particular, the group treated with 500 mg/kg showed a considerable increase in the relative volume of β pancreatic cells [31].

This final result was also confirmed by Yoon et al. (2011) who, when administering an OFI extract for 4 weeks to db/db mice and performing a histopathological analysis, observed that the morphology of the pancreatic islets were significantly improved [57].

In studies related to AO, the absorption of fat in the diet decreased through natural treatments, such as Litramine IQP-G-002AS (fiber derived from OFI). The results of four randomized clinical studies suggest that it is effective in promoting fat excretion and weight loss when taken at a daily dose of 3 g for seven days [58].

Another relevant study showed that an OFI extract included in a high-fat diet and administered for 12 weeks to C57BL/6 mice, prevented the rise of body weight and blood levels of LDL-Cho, HDL-Cho, tCho. In addition, the extract stimulated insulin secretion produced by their pancreatic islets [59].

Given that the information is very extensive, only the most significant documents will be analyzed, so the summary of the rest of the investigations, including in vitro and in vivo trials, clinical studies, and systematic reviews are included in Table 3.

Table 3. Main studies of *Opuntia* spp. on its effects in diabetes and obesity.

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
		Diabetes	
Clinical study	This is a pilot study where the effect of PPFs consumption on glucose metabolism was observed in 24 non-diabetic and non-obese men with FH.	The results showed a decrease in blood glucose (11%), insulin (11%), uric acid (10%) and TG (12%); while their body weight and HDL-Cho remained unchanged. This hypoglycemic action is related to an improvement in insulin sensitivity, and possibly to the pectin contained in PPFs.	[25]
In vivo	This study was on the intake of three doses of OHF (150, 250 and 500 mg/kg/day) regarding the regulation of blood glucose and hypolipidemic responses in diabetic rats induced by STZ.	After 7 weeks of oral treatment, fasting TG and blood glucose levels were significantly lower compared to the DM control group. In addition, the group treated with 500 mg/kg showed an increase in the relative volume of pancreatic β cells.	[31]
In vivo	The total phenolic content and the antioxidant activity of the seed oil of OdHw were analyzed using the Folin-Ciocalteu method and DPPH-scavenging assay, respectively. Also, the preventive effect of OdHw against alloxane (Allox)–induced DM was evaluated in albino mice.	The results showed that it had a high phenolic content and significant DPPH purifying activity. Likewise, the loss of body weight and the mortality rate caused by Allox decreased and the blood sugar level was controlled; which suggests that these protective actions are related to its phenolic content.	[35]
Clinical study	The hypoglycemic effect of *O. streptacantha* (OS) was evaluated in 16 patients with non-insulin-dependent diabetes mellitus (NIDDM).	Their serum glucose and insulin levels were quantified at 0, 60, 120 and 180 min. The result was that both parameters significantly decreased in individuals who ingested 500 g of roasted nopal stems. It was suggested to extend the studies in order to clarify the mechanism of action of OS.	[41]
Systematic review	The aim of this review was to identify the effects of *Opuntia* spp. consumption on glucose and insulin in humans, taking into account components such as PPFs, CLD and combined products.	During the research with six electronic databases, twenty articles were obtained (4 with PPFs, 12 with CLD and 4 with other products) that demonstrated a relevant reduction in serum glucose and insulin. The conclusions were that studies that specifically use PPFs or CLD have a high risk of bias. Apparently, PPFs have no significant effects on these parameters; unlike cladodes, which are more promising for hypoglycemic effects.	[42]
In vivo	Considering that only few data exist on OFI stem and fruit preparation combinations, the purpose of this study was to investigate the effects of an aqueous extract of CLD and a patented fruit stem/skin blend (ratio: 75/25) on blood glucose and plasma insulin in normal rats.	The observations were that the aqueous extract lowers glucose in a range of 6 and 176 mg/kg; while the patented blend was at 6 mg/kg. In addition, the mixture increases plasma insulin levels. The results suggest that both extracts have hypoglycemic activity, but the potential of the mixture is more significant as it shows a direct action on pancreatic β cells.	[43]
Clinical study	Considering the research of Van Proeyen et al. (63), the level of insulin stimulation by action of OFI combined with leucine (Leu) was compared. It was a randomized double-blind crossover study, where 11 subjects underwent an OGTT test after a cycling session. The study included an evaluation on whether this combination has an additive action on insulin stimulation after exercise.	After 60 min, the individuals ingested glucose and three types of capsules (some with 1000 mg OFI, others with a combination of OFI and Leu, and those with only Leu). Blood glucose and serum insulin were measured. The data showed that only the OFI group reduced blood glucose and the area under the glucose curve (AUGC). Furthermore, the OFI plus Leu group increased serum insulin concentration; suggesting that this combination may stimulate carbohydrate-induced insulin after doing exercise.	[44]

Table 3. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
In vivo	Berraaouan et al., calculated the in vitro antioxidant potential of OFI seed oil (CPSO) and its protective effect against Allox-induced diabetes mellitus.	They used a DPPH-scavenging assay for the first objective. To evaluate the preventive effect, Swiss albino mice treated with CPSO (2 mL/kg, orally) were used before and after Allox administration. During the in vivo test, body weight and fasting blood glucose were measured and a histopathological analysis of the pancreas took place. CPSO showed a relevant antioxidant action; it also reduced hyperglycemia and protected the islets of langerhans against Allox-induced tissue changes. The conclusion is that CPSO decreases oxidative stress and inhibits lesions in pancreatic β cells.	[45]
In vitro	The objective was to analyze some ethanol fractions of cladode polysaccharides of *O. monacantha* (POMC).	POMC fractions IV and V were obtained by means of anion exchange chromatography and purified with a Sephadex G-50 gel filtration column. By gel permeation chromatography (GPC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) it was established that POMC V had a molecular weight of 28.7 kDa and consisted mainly of rhamnose, arabinose and glucose; while POMC VI, had a smaller molecular weight (10.8 kDa) and was composed by rhamnose, mannose and glucose.	[46]
In vivo	The purpose of this study was to determine the most effective hypoglycemic component of OdHw polysaccharides and to study their antidiabetic ability in STZ-induced diabetic mice.	Initially, three types were identified (ODP-Ia, ODP-Ib, and ODP-II). After the administration of the ODP-Ia type, the food intake, blood glucose, and TG levels significantly decreased when measured in the fasting state. However, ODP-Ia did not increase insulin levels. It is suggested that ODP-Ia exerts its antihyperglycemic effect by protecting the liver and improving its sensitivity and cellular response.	[47]
In vivo	The aim of this research was to evaluate the effects of cactus pads extracts and pulp fruit on blood glucose concentration and the glycemic curve in Sprague-Dawley rats.	After 8 days of daily intake, the peaks and glycemic curves of the cactus pads and pulp fruit groups and the Cr (III) batch were less pronounced than those of the control group. In addition, a slight decrease in fasting blood glucose resulted. These data suggest that the Cr (III) content in these plant foods is related to their antihyperglycemic capacity.	[50]
In vivo	Ibañez and Meckes analyzed the hypoglycemic effect of a semi-purified fraction of OS in rabbits.	They confirmed that the powder fraction obtained produces an effect similar to the whole extract traditionally obtained from the stems of the vegetable. Their results suggest that the semi-purified product requires further evaluation to be considered a hypoglycemic agent.	[51]
In vivo	The antihyperglycemic effect of some edible plants *(Cucurbita ficifolia, Phaseolus vulgaris, OS, Spinacea oleracea, and Cucumis sativus)* was analyzed in healthy rabbits subjected to weekly tests of subcutaneous glucose tolerance.	Most of the plants had this capacity. However, OS was the one that most significantly decreased the area under the glucose tolerance curve and the hyperglycemic peak.	[52]
In vivo	Given that OdHw is traditionally used in the Canary Islands, the effect of its red juice on blood glucose levels in normoglycemic and Allox-induced diabetic rabbits was tested.	An oral dose of 5.0 mL/kg significantly reduced the increase in hyperglycemia in both types of rabbits. OdHw did not increase plasma insulin levels and was similar to that of an oral dose of tolbutamide (100 mg/kg). These data suggest that OdHw produces hypoglycemia mainly by reducing intestinal glucose absorption.	[53]

Table 3. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
In vivo	The study was on hypoglycemic activity of an purified extract of *O. fuliginosa* (Of) in induced diabetic rats by STZ.	Blood glucose and glycated hemoglobin (HbA1c) levels were reduced to normal values by a combined treatment of insulin and extract. When the insulin was with-drawn from the combination treatment, the extract maintained the normoglycemic state in the diabetic rats. The mechanism of action induced by the dose of Of (1.0 mg/kg/day) is possibly related to its fiber content.	[54]
In vivo	The purpose of the study was to evaluate the nutritional value of OdHw and its curative potential in STZ-induced diabetic rats.	The results showed that OdHw is a rich source of fiber, carbohydrates, minerals and vitamins. In addition, oral administration of OdHw juice significantly reduced blood glucose levels and by means of a histopathological analysis of pancreatic tissue improvement in the cells of the islets of Langerhans was observed, which may explain the antidiabetic effect of OdHw.	[55]
In vivo	The effects of some extracts of *O. Milpa Alta* [Aqueous, petroleum ether, ethyl acetate (EtOAc), butanol (BuOH)] were tested in STZ-induced diabetic mice.	The results indicated that all the extracts managed to lower glucose levels; although petroleum ether extract was the most significant.	[56]
In vivo	This study focused on the effect of OFI on blood glucose metabolism of db/db mice treated for 4 weeks.	After this period, food intake, plasma glucose and insulin levels decreased markedly. Furthermore, a histopathological analysis showed that the morphology of the pancreatic islets improved in the animals treated with OFI.	[57]
Systematic review	The purpose of the investigation was to analyze the efficacy of some natural products (*Opuntia, Gymnema, Tecoma, Ginseng, Karela, Alpha lipoic and Panaxans*) commonly used for diabetes.	After a MEDLINE search of articles published between 1960 and 2001, nopal was found to be the most widely used herbal hypoglycemic agent in people of Mexican descent; while Karela is mainly used in Asian countries. Studies reveal different mechanisms of action, among which the high content of soluble fiber stands out.	[60]
In vivo	The hypoglycemic activity of an extract of *O. lindheimeri Englem* was investigated in STZ-induced diabetic pigs.	A dose-dependent decrease in blood glucose concentration resulted from the oral administration of two doses (250 and 500 mg/kg) of the extract. Furthermore, the greatest hypoglycemic effect appeared 4 h after the intake. The conclusion is that this experimental model can be useful to evaluate long-term effects of *Opuntia* consumption given the physiological similarities of pigs with humans.	[61]
Clinical study	A double-blind controlled study (obese prediabetic individuals of both genders) was performed on the acute and chronic effects of OFI. The OGTT test was evaluated with a bolus of 400 mg of OFI ingested 30 min before consuming glucose.	In the acute phase, a significant decrease in blood glucose concentrations was observed during the next 60, 90 and 120 min. On the contrary, in the chronic phase, no differences were observed with the evaluated schedules of the OGTT, in the blood chemistry variables (insulin, adiponectin, Hb1Ac) and in the body composition after 16 weeks of supplying 200 mg of OFI.	[62]
In vivo	Two extracts of *O. streptacantha* [cladode traditional extract (LE) and traditional filtered sample (FE)] were evaluated in diabetic rats with STZ by two tests.	The first was to confirm its hypoglycemic capacity (LE 135 mg/kg and FE 27 mg/kg) and the second was to quantify the antihyperglycemic potential using oral glucose tolerance test (OGTT). The conclusion was that both extracts did not produce a significant hypoglycemic effect but an antihyperglycemic action compared to a control group of animals.	[63]

Table 3. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
Clinical study	Healthy men participated in a double-blind crossover study that included 2 experimental sessions. In the first one, they underwent OGTT at rest (OGTT-R) and cycling activity for 30 min. Immediately after the exercise, they received capsules containing 1000 mg of OFI extract and another OGTT (-EX) was performed.	Blood samples were collected at baseline and at 30-min intervals after the ingestion of 75 g of glucose in order to determine blood glucose and serum insulin. The results indicated that in OGTT-R, the AUGC was reduced by 26% and serum insulin had a higher concentration; while in OGTT-EX, glucose decreased approximately 10% lower with OFI compared to the placebo group. In conclusion, the extract can increase plasma insulin and facilitate the removal of an oral glucose load from the circulation at rest and after doing exercise.	[64]
In vitro In vivo	There is no information about whether the maturity stage in OFI can alter its antidiabetic capacity. Thus, the effect of small (SCF), medium (MCF) and large (LCF) cladode flours in diabetic rats was analyzed.	Only the MCF and SCF batches (50 mg/kg dose) showed a reduction in postprandial blood glucose. Furthermore, in vitro glucose diffusion tests showed a similar classification in both types of flour. It is considered was that the maturity stage alters the fiber content and produces differences in its viscosity, affecting in vitro and in vivo glucose responses.	[65]
		Obesity	
Systematic review	A sedentary lifestyle and excessive calorie consumption are known to be key factors in the prevalence of obesity. In consequence, reducing dietary fat absorption through approved drugs and natural treatments could help control this health problem.	Information gathered from four randomized controlled clinical studies on the efficacy of Litramine IQP-G-002AS (fiber derived from OFI) in reducing fat absorption suggests that it is effective in promoting fat excretion and weight loss; especially when ingested at a daily dose of 3 g for seven days.	[58]
In vivo	The purpose of this research was to determine the metabolic effect of an OFI extract in a diet-induced obese mouse model. The extract was added to a high-fat diet and administered for 12 weeks.	The doses used (0.3 and 0.6%) prevented the C57BL/6 mice from presenting high values of LDL-Cho, HDL-Cho, tCho and increasing their body weight. An improvement in glucose tolerance and an increase in energy expenditure were registered. In addition, the extract stimulated insulin secretion in isolated pancreatic islets. The decrease in metabolic abnormalities was associated with a higher content of mRNA for glucose transporter 2 (GLUT2) and peroxisome proliferator-activated receptor gamma (PPARγ).	[59]
In vivo	To determine the nutritional potential of whole *O. ficus-indica* seeds (OFIws) and its effect on food intake, Wistar rats received a treatment based on a diet supplemented with OFIws for nine weeks in which the efficiency of feed conversion, the protein efficiency index, and body weight were observed.	The results indicated a significant decrease in blood glucose concentration and body weight; as well as an increase in HDL-Cho and glycogen in the liver and skeletal muscle. Which suggests that OFIws is a healthy and useful food for obesity treatments.	[66]
Systematic review	Today, hundreds of weight loss products are in the global dietary supplement market. However, their effectiveness has not been fully proven. Through an electronic search, the effectiveness of PPFs was analyzed using published data from randomized clinical trials.	Five studies which varied in the design and quality of the reports were included. The analysis revealed a significant reduction in body mass index, body fat percentage, and tCho. Adverse events included gastric intolerance and flu symptoms. It is recommended to increase the number of clinical trials to have more consistent data.	[67]

Table 3. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
Systematic review	A bibliographic compilation was focused on the aspects of ethnobotany, toxicity, pharmacology, state of conservation, trade and chemistry of the medicinal plants used in Mexico, Central America and the Caribbean for the empirical treatment of obesity.	A total of 139 species were recorded, including *O. robusta* (OR), OM, OS, OM, OJ and OFI. The conclusions were: (a) There are no clinical studies in obese subjects using the medicinal plants mentioned in this review, (b) There are no herbal products approved in Mexico for the treatment of obesity, and (c) The need for other pharmacological, phytochemical, and toxicological studies with medicinal flora to obtain new antiobesity agents of high importance.	[68]

4.3. Hepatoprotective Effect

The liver plays a fundamental role in the regulation of various physiological processes, and its activity is related to different vital functions, such as metabolism, secretion and storage. It has the ability to detoxify endogenous and/or exogenous substances, helps in the metabolism of carbohydrates and fats, in the secretion of bile and participates in the supply of nutrients and energy. Unfortunately, "liver disease" (a term that indicates damage to cells, organ structure or function) continues to be one of the main threats to public health around the world. This damage can be induced by biological factors (bacteria, viruses and parasites), autoimmune diseases (immune hepatitis, primary biliary cirrhosis), as well as by the action of different chemicals, such as some drugs [high doses of acetaminophen (APAP) and anti-tuberculosis drugs], toxic compounds [carbon tetrachloride (CCl_4), thioacetamide, dimethylnitrosamine (DMN), D-galactosamine/lipopolysaccharide (GalN/LPS)], mycotoxins (aflatoxin B_1) and undoubtedly excessive alcohol consumption [12].

Despite the various therapeutic uses attributed to the genus *Opuntia,* scientific research on its hepatoprotective capacity began in 2004, when Wiese et al [69] reported that OFI could reduce the symptoms (nausea, dry mouth and anorexia) characteristic of hangover after consuming excess alcohol. Subsequently, Galati et al. (2005) examined the effects of prickly pear fruit (JPPF) juice against CCl_4-induced hepatotoxicity.

After administering 3 mL of JPPF per rat, the liver parenchyma lesion was restored after 72 h. In addition, plasma levels of ALT and AST were reduced. The investigators suggested that hepatoprotection could be related to flavonoids, betalains, and vitamin C, that synergistically, act on the antioxidant activity of JPPF [70].

On the other hand, Ncibi et al [71] demonstrated that a cactus cladode extract (CCE) from OFI could reduce the liver toxicity of the (CPF). Such conclusion resulted when combining the pesticide plus CCE and achieving significant normalization of biochemical parameters: ALT, AST, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), Cho and albumin (Alb), in contrast to animals treated with CPF only where the same parameters were notably affected. Research carried out by Dalel Brahmi's scientific group explored the protective potential of SCC against two hepatocarcinogenic agents in Balb/C mice: Benzo(a)pyrene [B(a)P] [72] and AFB_1 [73].

In both studies, the two carcinogens altered EOx markers, such as the level of malondialdehyde(MDA) and catalase activity (CAT), and increased the expression of heat shock proteins (Hsp 70 and Hsp 27). Likewise, the authors demonstrated that pre-treatment with SCC significantly decreased the oxidative damage induced in all the markers tested. In order to confirm the protective capacity of JPPF, in 2012, the team evaluated this property against liver injury induced by chronic consumption of ethanol (EtOH) in Wistar rats. Pretreatment of animals with EtOH plus JPPF (20 and 40 mL/kg body weight, orally) interestingly reduced biochemical markers of liver injury [such as ALT, AST, ALP, LDH, Cho, TG, and gamma glutamyl transferase (GGT)]. An improvement was also observed in lipid oxidation, glutathione (GSH) content, the activity of some antioxidant enzymes [such as SOD, CAT and glutathione peroxidase (GPx)], as well as histopathological lesions induced

by chronic ingestion of EtOH for 90 days. These results again suggest that this property could be attributed to its ability to end free radical chain reactions [74]. Considering that cyclophosphamide (CP) has a high toxicity associated with ROS overproduction, the ability to JPPF to reduce liver damage and cytotoxicity induced by this alkylating agent in mice was evaluated. Again, biochemical markers (AST, ALT, LDH, Alb) and EOx indicators [degree lipid peroxide (LPO) and MDA] were analyzed. Cytotoxicity was also studied by reducing nucleic acids, proteins, and glutathione in liver cells. After a pretreatment with JPPF, all mentioned liver markers were statistically restored [75].

Not only OFI has demonstrated a hepatoprotective potential, since González-Ponce et al. (2016) analyzed the antioxidant activity of OS and OR extracts against APAP-induced acute liver failure (ALF). They also administered both extracts (800 mg/kg/day, orally) to Wistar rats prior to APAP intoxication, which significantly attenuated lesion markers (AST, ALT, and ALP) and improved liver histology.

Furthermore, in a culture of hepatocytes, the extracts reduced LDH leakage and cell necrosis, both prophylactically and therapeutically. Apparently, OR showed higher levels of antioxidants than OS. These results suggested that both extracts could be considered as nutraceuticals to prevent ALF [76]. Finally and considering that OdHw appears to have antioxidant and anti-inflammatory properties, the hepatoprotective effect of a hydroalcoholic extract against lead acetate (Pb) -induced toxicity in an animal and cellular model was analyzed.

In the first case, Wistar rats received the extract (100 and 200 mg/kg/day) plus Pb for ten days. At the end of the period, it was observed that OdHw increased CAT activity and decreased the activity of MDA and serum liver enzymes (ALP, ALT, AST). In the case of the in vitro assay, HepG2 cells were used to measure three concentrations of OdHw (20, 40 and 80 μg/mL) on cell viability, MDA, GSH and TNF-α. The measurement confirmed that all concentrations reduced the levels of MDA and TNF-α and increased at GSH [77].

As can be seen, most of the studies analyzed suggest that the main mechanism of hepatoprotective action is to act on the inhibition of EOx. However, because the antioxidant process remains largely unknown, activation of factor 2 related to nuclear erythroid factor (Nrf2) has recently been explored. In this sense, Nakahara et al. [78] demonstrated that an OFI extract showed potent antioxidant activity through the activation of Nrf2. Their conclusion was made by confirming that the antioxidant capacity of OFI was canceled in Nrf2 knockdown keratinocytes.

Cells where Nad (p) h: Quinone oxidoreductase 1 (NQO1) inhibits the generation of ROS induced with B(a)P and TNF-α have been evaluated. The results suggested that OFI upregulated Nrf2-NQO1 through activation of the aryl hydrocarbon receptor (AHR) and AHR-OFI binding regulated the expression of epidermal barrier proteins (such as filaggrin and loricrin) [78].

Recently, INS-1 cells were exposed to Allox with different concentrations of polysaccharides extracted from *O. Milpa Alta* (MAP) to measure the Nrf2 pathway and the activation of apoptosis in response to an increase in EOx. MAP restored cell viability and SOD and GSH activity, while considerably decreased the release of ROS, LDH, MDA and nitric oxide (NO) levels. Possibly, MAP may attenuate the apoptosis induced by Allox by increasing the expression of Bcl-2, decreasing the expression of Bax and the activities of caspase-3 and caspase-9 [79].

4.4. Effects on Human Infertility

Globally, statistics suggest that approximately 15% of couples show concern when they want to conceive and fail after 12 months of regular unprotected sex. For a long time, the female gender was wrongly stigmatized for its inability to conceive, but scientific advances in human reproduction of the last four decades have associated approximately 50% of this infertility with the male gender. Different environmental, physiological, and genetic factors have been identified in "male factor" infertility, especially those related to sperm dysfunction [80].

Oligozoospermia is a disease characterized by low sperm count and quality and responsible of 90% of male infertility. Unfortunately, routine semen studies and analysis have shown that not all men who have normal parameters are fertile. The hidden factor is now known to be EOx and is recognized as a major cause of idiopathic male infertility.

The fact that sperm contain a large amount of unsaturated fatty acids makes them prone to lipid peroxidation, causing DNA damage and activating its apoptotic elimination through a p53-dependent and independent mechanism that can lead to infertility. Some studies have shown that disorders such as poor fertilization, pregnancy loss, birth defects, and poor embryonic development are associated with sperm damaged by excessive EOx [80–83].

In a normal physiological state, seminal plasma contains an antioxidant enzymatic mechanism capable of quenching ROS and protecting sperm. However, a high level of ROS, triggered by factors such as inflammation, DM, AO, alcoholism, smoking, and environmental pollutants can minimize this protective mechanism [80,84–87].

Different studies have suggested that some food supplements such as selenium, zinc, carnitine and arginine increase sperm count and motility; while antioxidants such as vitamin E, C and B12, carotenoids, coenzyme Q and glutathione are beneficial for the treatment of male infertility. In other words, these compounds can help in the balance between ROS generation and the protective enzymatic mechanism of sperm [80,88,89].

In the specific case of *Opuntia* spp., there is little research that has explored its action in human infertility. Meama et al. (2012) conducted the first study and tested the effect of OFI on sperm DNA fragmentation (SDF) exerted by cryopreservation in two sperm populations [PI (brighter) and PI (dimmer)]. Normozoospermic men underwent semen analysis for infertility and their cell samples were subsequently cryopreserved in the presence of OFI extracts.

The process induced an increase in SDF only in the PI sperm population. In contrast, the addition of OFI slightly reduced SDF without affecting cell viability. Their results suggest that OFI probably prevents some damage to sperm during a cryopreservation process [90]. Some studies also suggest that antioxidant treatments administered in high doses can block the oxidative processes essential for the compaction of sperm chromatin.

Consequently, a trial was performed in couples with infertility and at least 2 attempts at assisted reproductive technology (ART) to evaluate a nutritional support (called Condensyl™) of the cycle of a pure carbon without strong antioxidants. The treatment consisted of a combination of B vitamins, zinc, *Opuntia fig* extract, small amounts of N-acetylcysteine, and vitamin E. A group of 84 patients consumed 2 tablets of Condensyl™ per day for 12 months.

The final results showed a positive response rate of 64% for the decondensation index and 71% for the DNA fragmentation index. Thus, 18 couples achieved a pregnancy before the planned ART cycle. The rest of the couples underwent a new ART attempt resulting in 22 additional pregnancies and 15 live births. The conclusion with these data was that low doses of Condensyl™ may have a positive potential on fertility by achieving a pregnancy rate and a live birth rate of 70% and 57%, respectively [91].

Another significant study was the one designed by Hfaiedh et al. (2014) to investigate the protective capacity of CCE (100 mg/kg) on sodium dichromate (SD) -induced testicular damage in male Wistar rats. After a 40 day treatment with SCC, it was possible to restore serum testosterone level, sperm count and motility to levels comparable to the DS control group. A reduction in the elevated level of lipid peroxidation and a significant increase in testicular SOD, CAT, and GPx activities were also registered [92].

Probably, the effect of CCE to minimize the oxidative damage induced by SD motivated the development of another in vivo test; the purpose of which was to analyze the reversible antifertility potential of two doses (300 and 900 mg/kg) of a methanolic extract from the fruit of *O. elatior Mill* (OeM). After administering OeM for 60 days, epididymal sperm count and motility were reduced by 80% without a decrease in serum testosterone levels.

On the other hand, testicular steroidogenesis or libido were not affected, unlike male fertility, which was suppressed when they were mated with virgin female rats. Said suppression of fertility was dose-dependent and reached the 100% at the highest dose. However, withdrawal of treatment for two weeks recovered sperm count, serum testosterone levels, and fertility [93].

Recently, Akacha et al. (2020) determined the role of an OFI ethanolic extract (EEOFI) in methotrexate (MTX) -induced testicular damage in rats. They considered using this chemotherapeutic agent due to its various drawbacks, especially in cells that are constantly dividing and developing. EEOFI (0.4 g/kg) was administered to rats treated with MTX and subsequently the sperm were collected and quantified where their motility was determined.

They also evaluated EOx markers (MDA, CAT, GPx and SOD) and marked serum testosterone levels by radioimmunoassay. The results confirmed that EEOFI had protective effects on rat gonad histology, oxidative stress, and sperm count and motility. Furthermore, serum testosterone levels increased considerably. Their results also suggest that EEOFI improves testicular injury and has a potent stimulating effects on fertility [94]. The contradictory results opens the field of research to confirm the antioxidant action of *Opuntia genus* on fertility.

4.5. Chemopreventive and Antigenotoxic Effects

The chemopreventive and antigenotoxic potential of *Opuntia* spp. is another field of research that has been widely explored by various scientists. The concept of cancer chemoprevention "Use of natural or synthetic biologically active substances that can prevent, inhibit, or reverse tumor progression" was established in the 1970s, and in the specific case of these opuntioid cacti, there have been conducted 21 studies (mainly in vitro and in vivo tests) to date. In general, the results suggest various mechanisms of action to try to prevent the development of this disease.

It is important to remember that the transformation of normal to malignant cells is driven by a multi-step process (conceptually divided into initiation, promotion, progression, invasion, and metastasis) due to genetic alterations that include mutations and/or epigenetic changes caused by genotoxic agents or genotoxins; which by their origin are divided into physical, chemical and biological.

Observations on cancer etiology reveal that as many as 90–95% of carcinoma cases are associated with chemical agents, only 5–10% with physical agents and around 2–5% with biological agents. However, considering that all mutagens are genotoxic, but not all genotoxic substances are mutagenic, the compounds that reduce DNA damage caused by genotoxins are called antigenotoxic and/or antimutagenic agents [3,95].

In general, antimutagens have been classified as desmutagens and bioantimutagens. The first group considers substances that promote the elimination of genotoxic agents from the body, as well as substances that partially or totally inactivate mutagens by enzymatic or chemical interaction before the mutagen attacks DNA.

On the other hand, bioantimutagens (known as true antimutagens) can suppress the mutation process after DNA is damaged and act on the repair and replication processes; resulting in a decrease in the frequency of mutations [3]. The reality is that the mechanisms of action of antigenotoxic and/or chemopreventive agents are varied and complex and it would be very difficult to fully explain them in this document. In summary, observations report that they act in different cellular and molecular events including apoptosis, cell proliferation, cell cycle, EOx regulation, DNA repair, activation/detoxification of carcinogens by xenobiotic metabolizing enzymes, functional inactivation/activation of oncogenes and tumor suppressor genes, angiogenesis and metastasis [3,95].

Practically, the clinical study (the only one so far) developed by Palevitch et al. (1993) where they treated benign prostatic hypertrophy (BPH) with a dried flower preparation of OFI was the one that started in this field of research. Their results showed that patients treated with 2 capsules (250 mg of dried flowers/capsule) orally, three times a day, for 6 and 8 months, significantly improved the discomfort associated with BPH. However,

they were unable to establish the mode of action of this preparation [96]. Table 4 shows the main studies carried out with different plant parts of *Opuntia* spp. that have demonstrated chemopreventive and antigenotoxic potential. In summary, OFI is the most studied species in in vitro models and in different types of extracts [hexane, EtOAc, acetone, methanol (MeOH) and aqueous ones]. Likewise, hexane extracts of its seeds, extracts (aqueous and EtOAc) of its PPFs, juices of its different varieties of PPFs [red-purple (PPRP), white-green (PPWG) and yellow-orange (PPYO)] and some of its bioactive compounds such as betanin (betacyanin isolated from its PPFs) and isorhamnetin glycosides. Another species explored is OHF (mainly, in hexane extracts, aqueous and EtOAc); and to a lesser extent, OR extracts, polysaccharides extracted from OdHw and *O. microdasys* at post flowering stage F3 (OMF3). Different cell lines have been used in these studies; the most representative are those extracted from the cervix, bladder, ovarian cancer (OVCA420), immortalized normal ovarian cells (SKOV3), human chronic myeloid leukemia (K562), breast cancer (MCF-7), colon cancer (HT -29), human glioblastoma (U87MG), lung squamous carcinoma (SK-MES-1), human BJ fibroblasts, and Caco-2, SW480, and HeLa cancer cells.

In the case of in vivo tests, only mice (Balb/C and NIH) have been used, causing them genotoxic damage by administering some mycotoxins [zearalenone (ZEN) and AFB_1] or a mutagenic agent [such as methyl methanesulfonate (MMS)]. Skin cancer has also been induced by 7,12-dimethyl-benz [a] anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and ultraviolet B (UVB) photocarcinogenesis.

In general, the frequency of micronuclei (MN), chromosomal aberrations in bone marrow cells and DNA fragmentation were quantified. Together, the results of these studies suggest that the chemopreventive and/or antigenotoxic effect of OFI, OHF, OR, OdHw and OMF3 is related to their ability to inhibit cell proliferation and induce apoptosis, accumulate ROS (pro-oxidant activity), anticlastogenic potential, modulate lipid peroxidation, induce phase II detoxifying enzyme system and antioxidant capacity. Among the bioactive compounds that exhibited these capacities are flavonoids (such as quercetin, kaempferol, isorhamnetin), betalains (such as betanin and indicaxanthin), carotenoids and phenolic compounds [4,73,97–114].

Table 4. Main studies of *Opuntia* spp. on its chemopreventive and antigenotoxic potential.

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
In vitro In vivo	The purpose of the study was to evaluate the antioxidant capacity of three varieties of PPF juice [red-purple (PPRP), white-green (PPWG) and yellow-orange (PPYO)] in five different concentrations (100, 250, 500, 750 and 1000 mg/mL) by means of the DPPH method and selecting the variety with the highest antioxidant capacity to determine its anticlastogenic potential against MMS.	NIH mice were administered orally with PPRP and subsequently MMS was injected; which resulted in that PPRP was not a genotoxic agent, on the contrary, the reduction of MN frequency was proportional to the dose.	[4]
In vivo	The purpose of the study was to evaluate the antigenotoxic effect of CCE against AFB_1-induced damage in Balb/C mice. Animals were pretreated intraperitoneally with SCC (50 mg/kg body weight) for 2 weeks.	The results indicated that AFB1 induced significant alterations in EOx markers and was a genotoxic agent. In contrast, CCE reduced the number of chromosomal aberrations, DNA fragmentation, and the expression of p53 along with its associated genes (bax and bcl2). It is concluded that the genoprotective effect of CCE is probably related to its antioxidant capacity.	[73]
In vitro In vivo	In this study, the anticancer effect of five concentrations (0.5, 1.0, 5.0, 10 or 25%) of aqueous extracts of CLD from OFI in ovarian, cervix and bladder cells was evaluated; as well as in tumor growth in Balb/C mice.	After treating the cells for 3 and 5 days, an inhibition of cell growth and induction of apoptosis was confirmed in a dose-dependent and time-dependent manner. The extracts were also found to significantly suppress tumor growth and increase annexin IV expression in animals.	[97]

Table 4. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
In vitro	The antiproliferative potential of betanin isolated from PPFs from OFI on the human chronic myeloid leukemia cell line (K562) was analyzed.	The results showed a decrease in the proliferation of K562 cells treated with a concentration of 40 μM. On the other hand, scanning electron microscopy revealed apoptotic characteristics such as chromatin condensation, cell contraction, and membrane blistering. While flow cytometry (FCM) showed 28% of cells in G0/G1 phase. In conclusion, betanin can induce apoptosis through the intrinsic pathway.	[98]
In vivo	Zourgui et al., analyzed whether EOx is a relevant parameter in the toxicity induced by ZEN and evaluated the efficacy, safety and antigenotoxic capacity of CCE to prevent the deleterious effects of ZEN. Balb/C mice were treated with the mycotoxin and three doses (25, 50 and 100 mg/kg b.w.) of CCE from OFI.	The results showed that ZEN increased the level of MDA, CAT and the generation of protein carbonyls in kidney and liver. While from the lowest dose of CCE the oxidative damage induced by ZEN was reduced. On the other hand, the same toxin induced MN frequency and chromosomal aberrations in bone marrow cells. This phenomenon was reversed by the three doses of CCE; emphasizing that the highest dose of the extract was safe and did not induce any genotoxic effect. These data suggest that SCC may reduce the detrimental effects of EOx and ZEN-induced genotoxicity.	[99,100]
In vitro	Nine PPFs juices from OFI were characterized in terms of color, pH, acidity, phenolic content, flavonoids, and betalains. The study included its antioxidant activity in vitro against four cancer cell lines [mammary (MCF-7), prostate (PC3), colon (Caco-2) and hepatic (HepG2)].	In summary, the juices presented pH and acidity values that varied from 4.27 to 5.46 and from 0.03 to 0.27%, respectively. Variations were also observed in the content of flavonoids, betaxanthins and betacyanins. PC3 and Caco-2 were the cell lines most affected in their viability due to the action of PPF juices.	[101]
In vitro	Given that OHF has high concentrations of polyphenols and flavonoids, the anticancer effects of an EtOAc, aqueous and hexane extract on MCF-7 cells were investigated.	All extracts significantly decreased the number of viable cells in a concentration-dependent manner. Furthermore, a G1 arrest was induced in MCF-7 cells. In general, it was evidenced that the aqueous extract had a greater capacity to inhibit cell proliferation and induce apoptosis.	[102]
In vitro	Considering the previous studies of Yoon et al. (102) an extract of EtOAc, of hexane, and a fraction divided in water of *O. humifusa* (OHF) were again analyzed on cell proliferation, G1 arrest and apoptosis in U87MG human glioblastoma cells.	Cell proliferation was assessed using the MTT assay [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide], and the effects of each extract on cell cycle and apoptosis were analyzed by FCM. The results were that both the hexane extract and the aqueous fraction reduced the number of viable cells. Furthermore, cell arrest was again induced in G1.	[103]
In vitro	Ovarian cancer cells (OVCA420) and immortalized normal ovarian cells (SKOV3) were treated with two concentrations (5 and 10%) of an aqueous extract of prickly pear (AEPP) from OFI.	After 2 days of treatment, both types of cells treated with AEPP showed a relevant increase in ROS. Specifically, high levels of DNA fragmentation and the expression of genes related to apoptosis (Bax, Bad, caspase 3, Bcl2, p53 and p21) that are sensitive to ROS were also observed in OVCA420 cells. After three days of treatment, the expression of NF-kappa B decreased, while p-AKT increased. The conclusion was that the inhibitory effect of AEPP on cell growth is through the accumulation of ROS and induction of apoptosis.	[104]

Table 4. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
In vivo	Some in vitro tests have shown that OHF has anti-inflammatory, anti-proliferative and radical scavenging capabilities; Therefore, it was decided to evaluate its inhibitory effect on DMBA and TPA-induced skin cancer in Balb/C mice.	After previously feeding the animals with a diet containing 1.0 and 3.0% OHF, a reduction in the number of papillomas and epidermal hyperplasia occurred. The total antioxidant capacity, cutaneous glutathione S-transferase activity, and SOD also increased. Lipid peroxidation was measured in the skin cytosol and was only inhibited in the group fed 3% OHF. The results suggest that OHF exerts its chemoprevention by reducing EOx by modulating skin lipid peroxidation, enhancing antioxidant capacity, and inducing the phase II detoxifying enzyme system.	[105]
In vivo	The previous result of Lee et al. [105] that determined the chemopreventive capacity of OHF on skin cancer induced by DMBA and TPA motivated this new study to analyze the protective potential of OHF against UVB-induced photocarcinogenesis. Again, Balb/C mice were fed OHF and subsequently irradiated twice every week for 30 weeks.	The final evidence was that the diet inhibited UVB-induced epidermal hyperplasia, leukocyte infiltration, myeloperoxidase level, and pro-inflammatory cytokine levels. In addition, the presence of interleukin-1β (IL-1β), IL-6, TNF-α, the level of expression of mRNA and COX-2 were reduced. Taken together, these data suggest that such protection is associated with the inhibition not only of UVB-induced inflammatory responses involving COX-2 and pro-inflammatory cytokines, but also with the down-regulation of UVB-induced cellular proliferation.	[106]
In vitro	In this work, the residues from the juice production of PPFs derived from OFI and OR were explored as possible sources of natural chemotherapeutic ingredients against colon cancer. By means of a hydroalcoholic extraction and separation by adsorption, the natural extracts were produced and their antiproliferative effect was subsequently evaluated in the HT29 cell line (human colon carcinoma).	The results showed that the extracts inhibited cell growth and stopped the cell cycle, especially in G1, G2 and M. Betacyanins, ferulic acid and flavonoids (mainly isorhamnetin) are probably the main compounds responsible for cell cycle arrest. Besides, the death of cancer cells could have been induced by the pro-oxidant effect of these compounds.	[107]
In vitro	To analyze the antitumor effect of the polysaccharides extracted from OdHw on cells of squamous cell carcinoma of the lung (SK-MES-1), the AnnexinV assay, FCM and Western-blotting were used.	The results showed that different concentrations of polysaccharides inhibit the growth of SK-MES-1 cells and stop the cell cycle in phase S. The AnnexinV assay revealed the induction of apoptosis. These data suggest that cell inhibition and apoptosis may be attributed to an increased expression of the P53 protein and the tension homolog deleted on chromosome ten (PTEN) protein.	[108]
In vitro	The purpose of the research was to determine the antiproliferative effect of extracts of OFI and different isorhamnetin glycosides in two cancer cell lines (HT-29 and Caco-2).	The study showed that glycosides and extracts were more cytotoxic against HT-29 cells. A bioluminescent analysis revealed an increase in caspase 3/7 activity in cells treated with the extracts, while FCM confirmed that both extracts and glycosides induced greater apoptosis in HT-29 cells. However, isolated isorhamnetin was more apoptotic in the Caco-2 cell line. The conclusion was that glycosylation induces the antiproliferative effect exerted by isorhamnetin extracts and glycosides.	[109]

Table 4. *Cont.*

Type of Study	Objective and Characteristics	Results and Conclusion	Ref.
In vitro	The research was on the effect of an AEPP derived from OFI and its pigment Ind on the proliferation of Caco-2 cells.	Both compounds caused apoptosis in nutritionally relevant amounts and their action was dose-dependent. Despite this, Ind accounted for approximately 80% of the protective effect, although not inducing EOx in Caco-2 cells. Probably, the epigenomic activity of Ind was to demethylate the promoter of the tumor suppressor gene p16 and reactivate the expression of silenced mRNA, favoring cell inhibition in the G2/M phase.	[110]
In vitro	Initially, the bioactive compounds of different extracts (hexane, EtOAc, acetone, MeOH and MeOH: water) of cladodes from OFI were identified and quantified by HPLC. Subsequently, their chemopreventive activities were evaluated in two types of cells (MCF7 and SW480).	The results indicated that the acetone and MeOH extract showed the highest amount of polyphenolic compounds. Further to this, most of the extracts, with the exception of hexane, exhibited significant cytotoxicity in both cell lines; although the most sensitive was the SW480. These findings suggest that the cell death induced by the extracts caused an inhibition of cyclooxygenase-2 (COX-2) and increased the Bax/Bcl2 ratio, favoring apoptosis. The set of antioxidant, antiproliferative and proapoptotic activity of bioactive compounds probably promote their chemopreventive role.	[111]
In vitro	Considering that the total levels of polyphenol and ascorbic acid in OHF are high, the premise was that their antioxidant compounds could inhibit the survival of two cell lines [cervical carcinoma (HeLa) and human BJ fibroblasts].	Hexane extracts from their seeds and EtOAc extracts from PPFs and CLD significantly suppressed HeLa proliferation, but did not affect BJ fibroblasts. Another observation was that G1 phase arrest was induced in HeLa cells, which was associated with low levels of cyclin D1 [cyclin-dependent kinase 4 (Cdk4)]. This result motivated to examine the EtOAc extract on the tumor growth of the HeLa cell xenograft, due to the finding that the tumor volume had been reduced; which was correlated with the decrease in the expression of Cdk4 and cyclin D1. It is suggested that both extracts may be promising candidates for the treatment of human cervical carcinoma.	[112]
In vitro	This study involved two objectives; the first, to evaluate the analgesic and anti-inflammatory activity of OMF3; and the second, to determine its antigenotoxic effects in Allium cepa test.	By means of the acetic acid contortion test and the carrageenan-induced foot edema test, OMF3 showed to have high analgesic and anti-inflammatory activity (72 and 70%, respectively). OMF3 also induced an antimutagenic potential at a concentration of 60 µg/mL against H2O2-induced damage.	[113]
In vitro	Despite the fact that OFI is an important dietary source and a traditionally used medicinal plant, there are few studies on its toxic effects. Therefore, a toxicological evaluation was carried out using the 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazo-lium bromide (MTT), Comet and the γH2AX In-Cell Western Assay.	None of the extracts showed any cytotoxic or genotoxic effect on the HepG2 cell line; on the contrary, both the fruit pulp and the extracts of seeds, flowers and cladodes showed a protective effect against the genotoxicity induced by H2O2. This evidence suggests that OFI extracts do not have cytotoxic and/or genotoxic effects.	[114]

5. Conclusions and Perspectives

The investigations shown in this review demonstrate the nutritional, medicinal, pharmaceutical and preventive impact of the different species of *Opuntia* spp. However, they also reveal the possibility of expanding and conducting new studies (in vitro, in vivo and clinical) in order to confirm its different mechanisms of action that together favor its beneficial properties. In general, the antiatherogenic, antihyperlipidemic, antihypercholes-

terolemic and antidiabetic effect share two mechanisms of action; the first one related to the soluble fiber content that decreases body weight and slows down the speed of digestion and/or intestinal absorption of glucose and fatty acids. The second, undoubtedly, lies in its antioxidant property, which is directly related to the role that OXs plays in the development of atherosclerosis and cardiovascular diseases that are complications of DM2 and OA. This mechanism is related to the presence of some flavonoids, phenolic compounds and fatty acids, specifically quercetin 3-methyl ether and/or omega-6 linoleic acid from cactus seed oil, both with hypocholesterolemic effects. Likewise, betalains (such as indicaxanthin and betanin) protect the vascular endothelium from inflammation and cytokine-induced oxidative alteration, such as TNF-α. Also, the polysaccharide content has shown anti-inflammatory activity and the ability to reduce lipoperoxidation and/or sequester ROS. In the control of DM2, the hypothesis that the ingestion of nopal improves the postprandial glucose response and stimulates insulin secretion through a direct action on β pancreatic cells has also emerged.

In addition, the proposal that Cr (III), present in CLD, balances carbohydrate and lipid metabolism, favors a positive effect on insulin signaling and/or function, improves its systemic sensitization and reduces plasma glucose. After studies related to ASCVD, DM2 and OA, another widely explored field of research is its chemopreventive and antigenotoxic potential; where approximately 21 studies (mainly in vitro and in vivo tests) have suggested various mechanisms of action, highlighting the induction of apoptosis, inhibition of cell proliferation and cell cycle, activation of the phase II detoxifying enzyme system and DNA repair.

Furthermore, the antioxidant and/or regulatory effect of EOx has been included. These actions have also been considered as the main hepatoprotective mechanism of *Opuntia* spp. The antioxidant process has been analyzed for many years; yet, it remains largely unknown. So it would be interesting to increase research exploring the activation of Nrf2. In the case of the action of *Opuntia* spp. on human fertility, there is little research and the results are contradictory since some studies suggest that antioxidant treatments administered in high doses can block the oxidative processes essential for the compaction of sperm chromatin; which opens another field of investigation. Taken together, all the studies point to the conclusion that the CLD and PPFs from *Opuntia ficus-indica* (OFI) are the plant parts and the species that have been studied the most. Regarding the form of analysis, hexane, EtOAc, acetone, MeOH and aqueous extracts have been evaluated. Besides OFI, other species studied are OdHw, OHF, OS, OH, OA, OM and OR. It is convenient to remember that the Cactaceae family contains approximately 130 genera and 1500 species, which favors a wide genetic diversity that in conjunction with environmental conditions (climate, humidity), soil type, age of maturity of the cladodes and the harvest season generates differences in the phytochemical composition of their plant parts (PPFs, CLD, roots, flowers, seeds and stems) between wild and domesticated specie, inducing changes in its nutritional values and indisputably in its functional and/or therapeutic properties. Furthermore, although the public and some health care professionals believe that herbal medicines are relatively safe because they are "natural", there are remarkably little data to support this assumption. Therefore, *Opuntia* spp. species are not exempt from possible adverse and toxic effects. In general, OFI has been found to be well tolerated orally, even at high doses, presenting on certain occasions mild diarrhea, increased volume and frequency of stool, nausea, headache and low colonic obstruction [14,114,115]. Both aspects (genetic diversity and toxicology of *Opuntia* spp.) will be analyzed in more detail in part 2 of the manuscript.

In conclusion, by combining all the information in this review favors the field of research in the biotechnology area where new studies could be developed with other species to explore their capacities and pharmacological properties, their doses and administration intervals and analyze their possible toxic effects in the medium and long term. Likewise, the bioactive compounds extracted from the different species could be used in the preparation of drugs and nutraceuticals, as well as to obtain chemopreventive agents directed at cancer and/or chronic degenerative diseases. Future studies on different pure opuntiode

cacti, extracts and isolated bioactive compounds will allow a greater understanding of the properties of *Opuntia* spp., which is a genus of plant consumed by humans for more than 8000 years, with a high frequency throughout the world and which is apparently considered a safe plant.

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