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Special Issue Reprint

Phytochemicals in Plants

Recent Developments on the Occurrence, Composition, Stability, Health, Food and Pharmaceutical Applications—2nd Edition

Edited by
Nicoleta-Gabriela Hădărugă

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Phytochemicals in Plants: Recent Developments on the Occurrence, Composition, Stability, Health, Food and Pharmaceutical Applications—2nd Edition

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Guest Editor

Nicoleta-Gabriela Hădărugă

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About the Editor

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Article

Quantitative Determination of Biogenic Element Contents and Phytochemicals of Broccoli (*Brassica oleracea* var. *italica*) Cooked Using Different Techniques

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Abstract: In this study, the effect of different cooking techniques on broccoli moisture, total phenolic, total flavonoid, and radical scavenging capacity results, polyphenol contents, and their quantitative values was investigated. The total phenolic quantities of fresh and cooked broccoli samples were assessed to be between 36.32 (conventional boiling) and 423.39 mg GAE/100 g (microwave heating). The radical scavenging activities of the broccoli samples were reported between 2.55 (conventional boiling) and 4.99 mmol/kg (microwave heating). In addition, catechin and rutin quantities of the fresh and cooked broccoli samples were measured to be between 2.24 (conventional boiling) and 54.48 mg/100 g (microwave heating), and between 0.55 (conventional boiling) and 16.33 mg/100 g (microwave heating), respectively. The most abundant elements in fresh and cooked broccoli samples were K, Ca, P, S, and Mg. The results showed some changes depending on cooking techniques compared to the control. The bioactive properties of broccoli samples cooked by means of conventional boiling, boiling in vacuum bag, and high-pressure boiling were established to be lower compared to the fresh sample. Catechin, 3,4-dihydroxybenzoic acid, rutin, and gallic acid were the key phenolic compounds of fresh and cooked broccoli samples. The phenolic components of broccoli were significantly affected by the applied cooking techniques. The highest protein in broccoli samples was determined in the broccoli sample cooked by boiling in a vacuum bag. There were statistically significant changes among the mineral results of broccoli cooked with different cooking methods.

Keywords: broccoli; cooking methods; bioactive compounds; antioxidant capacity; polyphenols; elements; HPLC

1. Introduction

Broccoli (*Brassica oleracea* var. *italica*), which is native to the Eastern Mediterranean basin and Italy, and a member of the Brassicaceae family, is a horticultural and a favorite winter vegetable with high nutritional value, bioactive compounds, and antioxidant properties [1–5]. Phenolic compounds, phenolic acids such as kaempferol and ascorbic acid in broccoli have antioxidant capacity [6,7], Broccoli contains significant amounts of health-beneficial compounds, which has increased its consumption by people [8]. The consumption of broccoli has a positive effect on human health thanks to some minerals, phenolics, xanthophylls, sulforaphane, phenolics, and unique bioactive compounds [6]. Broccoli contains significant amounts of health-beneficial compounds, which has increased its consumption by people. Minerals are the building blocks of bones, teeth, blood, and muscle cells, and increase the usefulness of vitamins [9–11]. Phenolic compounds play a crucial role in exerting a wide variety of biochemical and pharmacological effects [12,13].

The phenolic components of foods, and therefore their potential antioxidant activities, are affected by domestic processing procedures such as cooking [14,15] and thermal treatments [16,17]. If oxidative enzymes are not inactivated during cooking, they can increase the chemical or enzymatic degradation of phenolic compounds or cause chemical changes that may affect quality properties [18,19]. Steaming has been reported to be more beneficial for certain health-promoting compounds in fruits and vegetables [20]. In addition to causing changes in the chemical composition of green leafy vegetables, cooking processes can also cause changes in the concentrations of bioactive compounds [21–24]. Among the main factors that can change the level of phytochemicals in vegetables before consumption, the most important ones are heat-involving preparation/processing, thermal degradation, oxidation, leaching, and matrix degradation [25]. Broccoli cooked with various cooking methods can also be used in salads or side dishes. Although the most common cooking methods are usually boiling and steaming, the use of sous vide has recently become widespread in order to preserve the content and increase the bioavailability of bioactive compounds in vegetables [20,26,27]. Various heat treatments are widely applied to green leafy vegetables, which are commonly consumed raw or cooked/processed, to neutralize microorganisms and enzymes and increase flavor, thus increasing product safety and quality [26,28]. Vegetables and fruits are foodstuffs that have an important place in nutrition. They contribute to human health not only with the vitamins and minerals they contain, but also with their phenolic components. Combined phytochemicals in plant foods act through various mechanisms such as antioxidant activity, cell regeneration, and tumor suppression [29,30]. Vegetables are consumed raw as well as processed and consumed as various products. Since it is difficult for vegetables to be stored for a long time without spoiling in fresh form, it is possible to store them for a long time by applying processes such as boiling and drying [31]. Boiling is applied to prevent enzymatic change in canned food production until heat treatment, to prevent the negative effects of enzymes in the drying process until the end of drying, and to prevent the effect of enzymes until consumption in freezing preservation. Enzymes are rendered inactive by these processes. Thus, the raw material is prevented from undergoing enzymatic changes until it is sterilized. In addition, the microorganism load is also reduced by boiling [32]. Drying is one of the first preservation methods used by humanity to preserve food. Today, in parallel with the increasing consumption trend of ready-made foods, the importance of dried vegetables, which is one of the basic ingredients of such foods, is increasing all over the world. Although vegetables are generally consumed as fresh in Turkey, vegetables dried by various methods are also demanded by final consumers and food industry companies. The dried vegetable sector has become one of the important sub-sectors of the food industry with the modern drying methods it uses as well as the traditional sun drying method [30,33]. Boiling and steaming are the most widely used traditional cooking methods. Traditional cooking methods can lead to a loss of nutrients and flavor elements [34]. New cooking methods are being studied to minimize these losses. The objective of this investigation was to monitor the effect of different cooking techniques on broccoli moisture, total phenolic, total flavonoid, and radical scavenging capacity results, polyphenol contents, and their quantitative values.

2. Results and Discussion

2.1. Total Phenolic and Total Flavonoid Amounts, and Antioxidant Activity Values of Broccoli Cooked with Different Cooking Techniques

The moisture quantities and bioactive properties of broccoli florets cooked with different cooking techniques are displayed in Table 1. The results showed some changes depending on cooking techniques compared to the control. The moisture quantities of fresh and cooked broccoli samples were found to be between 50.84 (microwave) and 91.82% (conventional boiling). In general, the moisture quantities of broccoli cooked by means of conventional boiling (open pot), boiling in a vacuum bag, and high-pressure boiling were established to be higher when compared to fresh, while the moisture amounts of broccoli

samples cooked with conventional heating and microwave heating application are found to be lower than those of fresh ones.

Table 1. Bioactive properties of broccoli cooked using different techniques.

Process	Moisture Content (%)	Total Phenolic Content (mg/100 g)	Total Flavonoid Content (mg/100 g)	Antioxidant Activity (mmol/kg)
Fresh	86.93 ± 0.09 d *	116.69 ± 0.74 c	157.30 ± 5.06 b	4.75 ± 0.02 c
Conventional boiling	91.82 ± 0.50 a **	36.32 ± 0.46 f	60.16 ± 0.90 f	2.55 ± 0.10 f
Boiling in a vacuum bag	89.58 ± 0.05 c	75.63 ± 1.24 d	92.86 ± 0.78 d	3.75 ± 0.05 d
High-pressure boiling	90.41 ± 0.70 b	65.05 ± 1.95 e	73.81 ± 0.78 e	3.60 ± 0.04 e
Conventional heating	72.84 ± 0.68 e	148.04 ± 4.32 b	147.78 ± 2.50 c	4.99 ± 0.00 a
Microwave heating	50.84 ± 1.81 f	423.39 ± 3.96 a	409.68 ± 3.67 a	4.79 ± 0.00 b

* Standard deviation; ** values within each column followed by different letters are significantly different: "$p < 0.05$".

While the total phenolic amounts of cooked broccoli samples were found to be between 36.32 (conventional boiling) and 423.39 mg GAE/100 g (microwave heating), the total phenolic quantity of fresh broccoli samples was 116.69 mg GAE/100 g. Also, the total flavonoid quantity of fresh broccoli samples was recorded as 157.30 mg/100 g, while the total flavonoid quantities of cooked broccoli samples ranged between 60.16 (conventional boiling) and 409.68 mg/100 g (microwave heating). In addition, the antioxidant activities of fresh and cooked broccoli samples were assessed to be between 2.55 (conventional boiling) and 4.99 mmol/kg (microwave heating). While the total phenol and total flavonoid quantities and the antioxidant activities of broccoli samples cooked by means of conventional boiling, boiling in a vacuum bag, and high-pressure boiling were found to be lower compared to the fresh sample, the total phenol, total flavonoid, and antioxidant activities of broccoli samples cooked in conventional heating and microwave heating were established to be higher than the results of both fresh broccoli and broccoli cooked using the other three cooking techniques. The fact that the total phenol and total flavonoid quantities, and the radical scavenging capacity values of broccoli samples cooked using conventional and microwave heating were higher than the others is probably caused by Maillard reaction products that may occur as a result of dry heating. The amount of total phenols and flavonoids in broccoli samples cooked by means of boiling in a vacuum bag, high-pressure boiling, and conventional boiling may have decreased due to the deterioration of their structure. There were statistically significant changes among the bioactive characteristics of broccoli cooked with different cooking methods ($p < 0.05$). Many physical and chemical changes and thermal deteriorations may occur in the structure of most vegetables cooked using boiling, microwave oven, steaming, or baking methods [35]. In addition, Maillard reaction products, formed as a result of heat treatment, can produce stronger antioxidant products [36,37]. The moisture contents of fresh broccoli, water-boiled broccoli, steamed broccoli, and microwaved broccoli were 89.86%, 93.26%, 91.87%, and 93.27%, respectively [38]. The protein contents (dw) of fresh broccoli, water-boiled broccoli, steamed broccoli, and microwaved broccoli were 3.34%, 2.27%, 2.68%, and 2.26%, respectively [38]. In addition to these, the total phenolic content of broccoli ranged from 412 to 987 mgGAE/100 g in fresh samples [34,39]. The total phenolic contents of raw broccoli and broccoli cooked using MW, boiling, and steaming were 169.6 mg GAE/100 g, 164.3–185.8 mgGAE/100 g, 164.2–171.3 mgGAE/100 g, and 67.9–139.3 mgGAE/100 g, respectively [40]. The total phenolic content and radical scavenging activity of raw broccoli were found to be 2282.97 mgGAE/kg and 0.189 mmolTE/g, respectively [5]. The total phenolic result and radical scavenging activities of raw broccoli cooked using the sous vide method at different times (5, 10, and 15 min.) ranged between 1845.88 and 2098.96 mg GAE/kg, and between 0.119 and 0.158 mmolTE/g, respectively [5]. The total phenolic and antioxidant activity results of raw broccoli cooked using the steaming method at different times (5, 10, and 15 min.) ranged between 1981 and 2188.3 mg GAE/kg, and between 0.144 and 0.174 mmolTE/g, respectively [5]. The total phenolic content and antioxidant activities

of raw broccoli cooked using the boiling method at different times (5, 10, and 15 min.) ranged between 926.56 and 1692.47 mgGAE/kg, and between 0.069 and 0.133 mmolTE/g, respectively [5]. The total phenolic content of vegetables decreased using both conventional and sous vide cooking methods [41]. The antioxidant activity values of raw broccoli and broccoli cooked using MW, steaming, and boiling were 637, 563–692, 599–732, and 249–617 μmol TE/100 g, respectively [40]. Turkmen et al. [42] pointed out that the cooking method with the highest total phenol content of broccoli was microwave cooking. In a previous study, there were no differences in antioxidant activity between fresh and cooked broccoli [43]. It has been stated that there are differences even in the antioxidant capacity results of different parts of broccoli [44,45]. Findings pointed out some fluctuations compared to the results of several studies. These changes in results are probably due to the genetic variation of the sample, different climatic factors, plant parts, boiling times and types, agronomical conditions, harvest time, variety, and analytical conditions such as solvent used and extraction types.

2.2. The Phenolic Compounds of Fresh and Cooked Broccoli Samples

The phenolic profiles and their quantitative results of fresh and cooked broccoli samples are displayed in Table 2. Catechin, 3,4-dihydroxybenzoic acid, rutin, and gallic acid were the key phenolic compounds of fresh and cooked broccoli samples (Figure 1). It was observed that the phenolic components of broccoli were significantly affected by the applied cooking techniques. It was determined that the amount of phenolic components of cooked broccoli significantly decreased when compared to the fresh sample. However, the amounts of some phenolic profiles differed depending on the cooking technique. The phenolic components of broccoli samples cooked with conventional heating and microwave heating were higher when compared to the results of fresh broccoli and broccoli cooked using other cooking techniques. While the gallic acid quantities of broccoli florets varied between 0.52 (conventional boiling) and 4.29 mg/100 g (microwave heating), the 3,4-dihydroxybenzoic acid quantities of broccoli samples were assessed to be between 0.41 (conventional boiling) and 15.17 mg/100 g (microwave heating). In addition, the catechin and rutin quantities of fresh and cooked broccoli samples were assessed to be between 2.24 (conventional boiling) and 54.48 mg/100 g (microwave heating), and between 0.55 (conventional boiling) and 16.33 mg/100 g (microwave heating), respectively. The highest caffeic (7.07), syringic (6.21), *p*-coumaric acid (2.03), ferulic acid (4.72), resveratrol (1.11), quercetin (4.68), cinnamic acid (0.78), and kaempferol (5.12 mg/100 g) were found in broccoli cooked in the microwave. A significant part of the phenolic compounds in broccoli were adversely affected by conventional boiling, boiling in a vacuum bag, and high-pressure boiling methods. The catechin contents of broccoli cooked by means of boiling in a vacuum bag, high-pressure boiling, and conventional boiling were higher than broccoli cooked using conventional boiling. There were statistically significant changes among the phenolic compounds of broccoli cooked with different cooking methods ($p < 0.05$). Gunathilake et al. [46] pointed out that there is a decrease in total polyphenolic compounds during the cooking of some vegetables, and this decrease is probably due to the diffusion of polyphenolic compounds into boiling water. The effect of cooking methods on the release of phenolic constituents showed that heat treatments cause a partial hydrolysis of conjugated polyphenols. The release of polyphenols into free phenolic compounds triggered by heat treatment has been reported [23,35]. Caffeic acid, chlorogenic acid, and neochlorogenic acid were the major phenolic acids found in broccoli [45,47]. Fresh broccoli and broccoli cooked in the microwave contained 1.845 and 0.173 μg/g quercetin, 1.230 and 0.474 isorhamnetin, 4.976 and 7.252 *trans*-ferulic acid, 0.262 and 1.258 *p*-coumaric acid, and 52.158 and 46.489 μg/g chlorogenic acid, respectively [48]. Lopez-Hernandez et al. [40] reported that 0.27 mg/kg gallic acid, 16.43 chlorogenic acid, 0.97 caffeic acid, 10.89 isoquercitrin, 0.16 myricetin, 0.30 luteolin, and 0.12 quercetin were identified in raw broccoli. Also, 0.28–0.32 and 0.29–0.35 mg/kg gallic acid, 11.97–18.76 and 15.25–25.84 chlorogenic acid, 0.72–1.06 and 0.93–1.27 caffeic acid, 10.49–11.16 and 9.87–12.86 isoquercitrin, 0.14–0.18 and

0.14–0.18 myricetin, 0.31–0.37 and 0.39–15.01 luteolin, and 0.10–0.13 and 0.11–0.15 mg/kg quercetin were detected in broccoli cooked using MW and steaming, respectively [40]. In another study, adequate amounts of chlorogenic, neochlorogenic, and ferulic acids were detected in three broccoli samples (stem, leaf, and flower), while caffeic and *p*-coumaric acids were measured in broccoli leaf extracts. In addition, gallic acid and vanillic acid were detected in broccoli stems and flower extracts, while sinapic acid was detected only in broccoli leaves and flower extracts [49]. Our findings illustrated some fluctuations compared to the results of previous studies. These changes are likely due to broccoli variety, agricultural and climatic factors, harvest time, used parts, solvent types, extraction methods, and some other factors such as analytical conditions, storage, and cooking times and types.

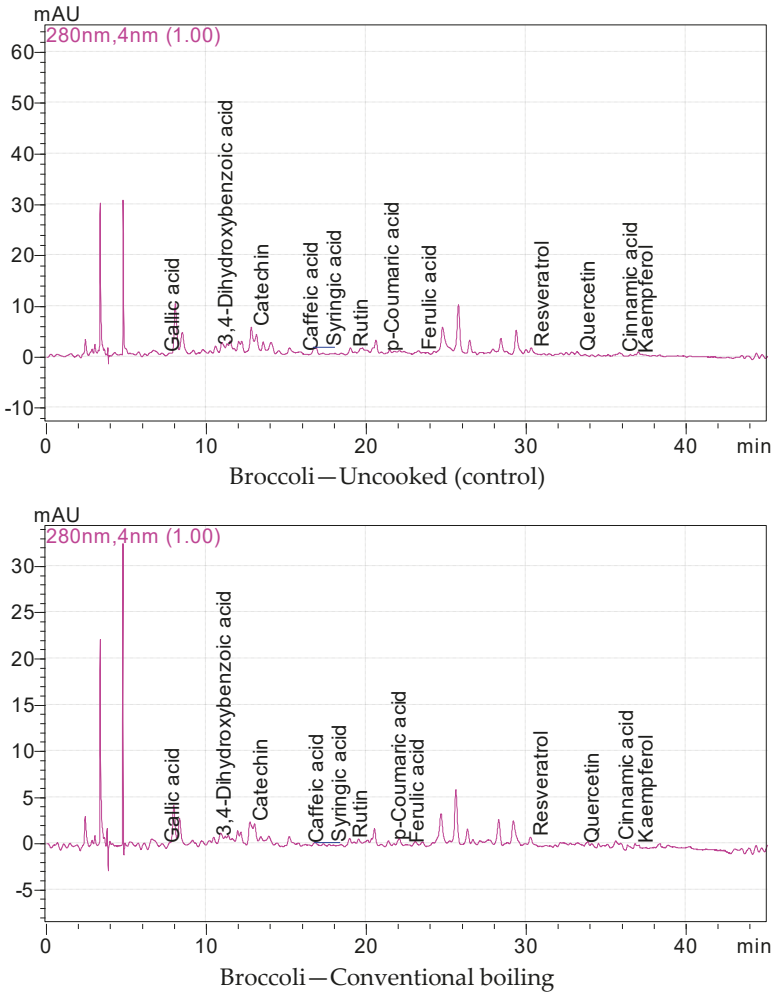


Figure 1. *Cont.*

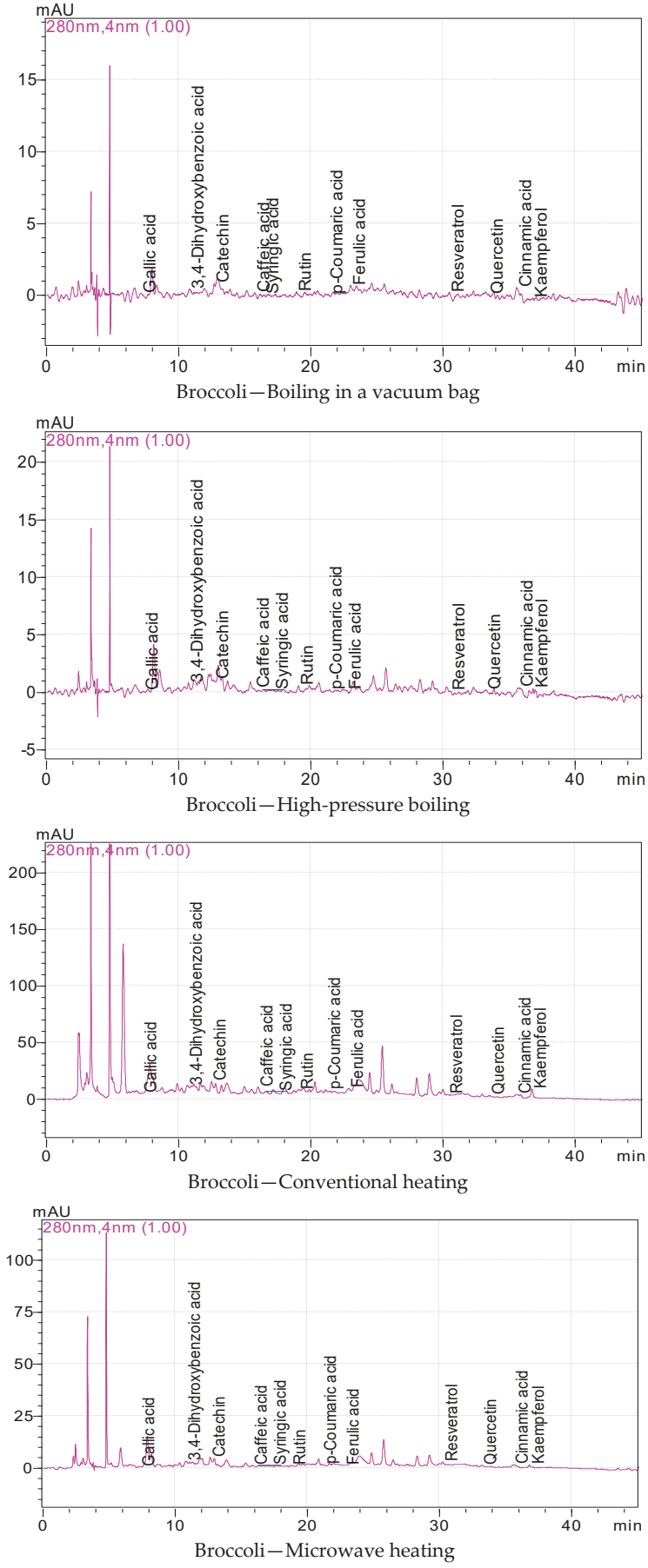


Figure 1. Phenolic chromatograms of broccoli samples.

Table 2. Phenolic compounds of broccoli cooked using different techniques.

Phenolic Compounds (mg/100 g)	Fresh	Conventional Boiling	Boiling in a Vacuum Bag	High-Pressure Boiling	Conventional Heating	Microwave Heating
Gallic acid	1.53 ± 0.43 c *	0.52 ± 0.01 f	1.91 ± 0.57 b	1.02 ± 0.36 d	0.66 ± 0.22 e	4.29 ± 0.94 a
3,4-Dihydroxybenzoic acid	3.48 ± 0.36 b **	0.41 ± 0.10 f	2.13 ± 0.26 d	1.77 ± 0.22 e	2.83 ± 0.91 c	15.17 ± 0.21 a
Catechin	24.00 ± 2.11 b	2.24 ± 0.49 f	10.93 ± 1.48 d	7.01 ± 1.43 e	13.36 ± 2.69 c	54.48 ± 0.75 a
Caffeic acid	0.83 ± 0.30 b	0.13 ± 0.02 d	0.15 ± 0.06 c	0.06 ± 0.0 e	0.86 ± 0.36 b	7.07 ± 0.17 a
Syringic acid	0.60 ± 0.06 c	0.08 ± 0.02 e	0.19 ± 0.02 d	0.16 ± 0.04 d	0.92 ± 0.49 b	6.21 ± 0.17 a
Rutin	4.16 ± 0.77 b	0.55 ± 0.06 f	1.29 ± 0.17 d	0.62 ± 0.14 e	2.56 ± 0.69 c	16.33 ± 1.58 a
p-Coumaric acid	0.21 ± 0.06 e	0.02 ± 0.00 f	0.08 ± 0.01 d	0.06 ± 0.01 e	0.36 ± 0.14 b	2.03 ± 0.06 a
Ferulic acid	0.21 ± 0.04 d	0.07 ± 0.03 f	0.12 ± 0.02 e	0.26 ± 0.09 c	0.59 ± 0.27 b	4.72 ± 0.21 a
Resveratrol	0.44 ± 0.03 b	0.07 ± 0.01 e	0.31 ± 0.01 c	0.13 ± 0.03 d	0.46 ± 0.08 b	1.11 ± 0.22 a
Quercetin	0.67 ± 0.04 b	0.62 ± 0.11 c	0.50 ± 0.08 d	0.40 ± 0.05 e	0.38 ± 0.10 f	4.68 ± 0.52 a
Cinnamic acid	0.16 ± 0.03 d	0.15 ± 0.03 d	0.09 ± 0.03 e	0.30 ± 0.04 c	0.33 ± 0.04 b	0.78 ± 0.04 a
Kaempferol	0.23 ± 0.06 e	0.31 ± 0.08 d	0.21 ± 0.06 ef	0.51 ± 0.06 c	0.74 ± 0.02 b	5.12 ± 0.47 a

* standard deviation; ** values within each row followed by different letters are significantly different at $p < 0.05$.

2.3. Mineral and Protein Contents of Broccoli Cooked with Different Cooking Techniques

The mineral and protein results of fresh and cooked broccoli samples are depicted in Table 3. The most abundant elements in fresh and cooked broccoli samples were K, Ca, P, S, and Mg. The element with the highest amount among the microelements was Fe, followed by Zn, Mn, B, and Cu in decreasing order. While the P amounts of fresh and cooked broccoli samples ranged between 3114.94 (fresh) and 4383.36 mg/kg (conventional boiling), the K quantities of broccoli samples were assessed to be between 19,638.27 (conventional boiling) and 32,729.06 mg/kg (boiling in vacuum bag). Also, the Ca and Mg amounts of fresh and cooked broccoli samples were found to be between 3555.67 (fresh) and 6052.mg/kg (conventional boiling), and between 1197.02 (fresh) and 5039.27 mg/kg (conventional heating), respectively. In addition, the S quantities of fresh and cooked broccoli samples varied between 3167.03 (conventional boiling) and 5112.97 mg/kg (microwave heating). Looking at the microelements, the Fe and Zn quantities of fresh and cooked broccoli samples were assessed to be between 41.46 (boiling in vacuum bag) and 55.99 mg/kg (conventional heating), and between 11.88 (fresh) and 17.53 mg/kg (conventional heating), respectively. Also, while the Cu results of fresh and cooked broccoli samples ranged between 2.56 (conventional heating) and 3.55 mg/kg (boiling in vacuum bag), the Mn amounts of broccoli samples were measured to be between 9.61 (fresh) and 15.22 mg/kg (high-pressure boiling). In general, the mineral content of broccoli samples cooked by different methods increased when compared to the control (fresh). Partial reductions were observed in some of the applied cooking techniques. The protein contents of fresh and cooked broccoli samples ranged between 13.35% (fresh) and 17.27% (boiling in a vacuum bag). Therefore, the highest protein in broccoli samples was determined to be in the broccoli sample cooked by means of boiling in a vacuum bag, followed by the sample cooked using conventional boiling, high-pressure boiling, microwave heating, conventional heating, and the fresh sample in descending order. There were statistically significant changes among the mineral results of broccoli cooked with different cooking methods ($p < 0.05$). Broccoli is a good source of elements such as Ca, Mg, Na, K, Ca, Cl, P, and S, and trace elements such as Fe, Zn, Mn and Cu, which are essential for human nutrition [7,50]. Fresh broccoli, water-boiled broccoli, steamed broccoli, and microwaved broccoli contained 8.67, 8.01, 8.11, and 8.61 mg/100 g Zn; 2.66, 1.68, 2.33, and 1.78 mg/100 g Fe; 112.52, 28.52, 94.21, and 63.79 mg/100 g Ca; 562.22, 275.37, 447.72, and 205.20 mg/100 g Mg; 3992.2943.76, 3796.3, and 2460.02 mg/100 g K; 576.52, 235.45, 379.58, and 256.61 mg/100 g Na; respectively [38]. Fresh broccoli contained 562.22 Mg, 3992.4 K, 576.52 Na, 8.67 Zn, 2.66 Fe, and 112.52 Ca mg/100 g (dw) [38]. Our results showed some differences with results described by Farnham et al. [50], Mukherjee et al. [7], and Mansour et al. [38], who stated that broccoli is a good alternative source of Ca, K, and Na. It was thought that the bioactive properties, phenolic compounds, and mineral and protein quantities of fresh and cooked broccoli samples probably vary depending on growing conditions, the harvest time of broccoli, soil plant nutrient elements, cooking techniques, cooking time, and analytical conditions.

Table 3. Mineral (mg/kg) and crude protein (%) contents of broccoli cooked using different techniques.

Treatments	P	K	Ca	Mg	S	Fe	Cu	Mn	Zn	B	Protein
Control (Fresh)	3114.94 ± 21.59 f	29,730.49 ± 299.48 c *	3555.67 ± 54.46 f	1197.02 ± 50.90 f	3935.51 ± 34.56 e	46.60 ± 1.40 e	2.73 ± 0.03 d	9.61 ± 0.55 f	11.88 ± 0.11 f	2.76 ± 0.04 d	13.35 ± 0.32 f
Conventional boiling	4383.36 ± 159.37 a	19,638.27 ± 526.42 f **	6052.69 ± 150.03 a	1610.79 ± 37.84 b	3167.03 ± 43.75 f	51.59 ± 1.14 c	3.28 ± 0.34 b	13.07 ± 0.54 e	13.48 ± 0.09 e	2.21 ± 0.41 e	15.75 ± 0.43 b
Boiling in a vacuum bag	3787.59 ± 30.86 d	32,729.06 ± 998.34 a	4731.34 ± 240.78 c	1508.55 ± 30.02 c	4208.92 ± 60.08 c	41.46 ± 0.24 f	3.55 ± 0.10 a	14.54 ± 0.05 d	16.30 ± 0.02 b	6.13 ± 0.72 a	17.27 ± 0.47 a
High-pressure boiling	3908.20 ± 16.18 c	21,876.31 ± 463.36 e	5598.51 ± 32.73 b	1326.53 ± 0.88 e	4015.41 ± 19.26 d	53.06 ± 2.11 b	2.97 ± 0.40 c	15.22 ± 0.13 a	14.34 ± 0.12 d	1.70 ± 0.02 f	15.61 ± 0.12 c
Conventional heating	3658.44 ± 85.05 e	28,322.47 ± 1190.18 d	3791.24 ± 127.93 e	5039.27 ± 557.90 a	4969.11 ± 137.71 b	55.99 ± 5.31 a	2.56 ± 0.11 f	14.89 ± 1.05 c	17.53 ± 1.16 a	5.12 ± 0.22 b	13.85 ± 0.83 e
Microwave heating	3934.30 ± 51.88 b	30,420.44 ± 211.57 b	4156.85 ± 73.95 d	1392.06 ± 42.53 d	5112.97 ± 70.60 a	48.51 ± 0.36 d	2.64 ± 0.13 e	15.04 ± 0.71 b	15.39 ± 1.71 c	4.41 ± 0.68 c	14.02 ± 0.80 d

* standard deviation; ** values within each column followed by different letters are significantly different at $p < 0.05$.

3. Material and Methods

3.1. Material

Broccoli samples were purchased from a local market in Konya province in Turkey. The samples were brought in cool conditions to the laboratory, washed, and divided into florets. The broccoli florets had stems and were approximately 3–4 cm wide and 6–7 cm long.

3.2. Methods

3.2.1. Boiling and Heating Processes

Broccoli samples were cooked using conventional boiling, boiling in a vacuum bag, a pressure cooker, conventional heating, and microwave heating for 13, 13, 7, 10, and 10 min. In the pressure cooker, 7 min was taken into account as the cooking time from the steam exit. In the oven, after the temperature was adjusted to 200 °C, a cooking time of 10 min was applied. The sous vide process was carried out using 100 g broccoli in plastic packaging at 100 °C/10 min. Conventional boiling was carried out in an open pot. Also, broccoli samples were heated in a microwave at 720 W for 10 min.

3.2.2. Determination of Moisture Results of Broccoli Samples

The moisture results of broccoli samples were recorded using the KERN & SOHN GmbH infrared moisture analyser.

3.2.3. Determination of Protein Quantities of Broccoli Samples

The protein contents of the broccoli samples were established according to the AOAC [51] method.

3.2.4. Extraction Procedure

Broccoli samples were extracted according to the study recognized by Girgin and El [52]. After 3 g powdered broccoli samples was added to 20 mL of solvent (methanol–water, 80:20, v/v), the solution was stored in an ultrasonic bath for 30 min. Then, it was centrifuged for 10 min. The supernatant was removed and these steps were carried out twice with 20 mL of solvent. The combined extracts were used for analyses.

3.2.5. Total Phenolic Results of Broccoli Samples

The Folin–Ciocalteu (FC) reagent was used to determine the total phenolic contents of broccoli extract according to Yoo et al. [53]. FC (1 mL) and Na_2CO_3 (10 mL) were added to the extract and mixed using a vortex mixer. Deionized water was added until the final volume was 25 mL, and the sample was kept in darkness for 1 h. After pre-processing, the absorbance value of the sample was recorded at 750 nm. The findings are stated as mg gallic acid equivalent/100 g.

3.2.6. Total Flavonoid Content of Broccoli Samples

The total flavonoid results of broccoli samples were obtained according to the work recognized by Hogan et al. [54]. After pre-processing, the absorbance result of the mixture was measured at 510 nm. The findings obtained are given as mg quercetin (QE)/100 g.

3.2.7. Antioxidant Activity Results of Broccoli Samples

1.1-diphenyl-2-picrylhydrazyl (DPPH) was applied for the antioxidant activity results of broccoli extracts [55]. The extract was added to 2 mL of a methanolic solution of DPPH, and was then vortexed and kept in darkness for 30 min; the absorbance of extracts was obtained at 517 nm. The results obtained are given as mmol trolox (TE)/kg.

3.2.8. Phenolic Compounds

A High Performance Liquid Chromatography unit mounted on a PDA detector and an Inertsil ODS-3 (5 μm; 4.6 × 250 mm) column were used for the chromatographic separation of the phenolic compounds of broccoli samples. The injection volume was 20 μL. The peaks

were recognized at 280 using a PDA detector. The mobile phase was a mixture of 0.05% acetic acid in water (A) and acetonitrile (B) with a flow rate of 1 mL/min at 30 °C.

3.2.9. Determination of Minerals of Broccoli Samples

After 0.5 g broccoli powder, dried at 70 °C, was incinerated by using 5 mL of 65% HNO_3 and 2 mL of 35% H_2O_2 in a closed microwave, its volume was completed with 20 mL distilled water. Minerals were measured using ICP-AES [56].

3.3. Statistical Analyses

The JMP version 9.0 statistical analysis method was used for the analysis of variance. The mean of the triplicate analysis data was subjected to analysis of variance. The significant differences among the values of control and cooking types were determined using Duncan's Multiple Range Test ($p < 0.05$).

4. Conclusions

Cooking methods affect the bioactive substances, phenolic profiles, mineral result, and antioxidant activity observed in broccoli. The total phenol, total flavonoid, and radical scavenging capacity results of broccoli samples cooked using conventional boiling, boiling in a vacuum bag, and boiling in a steam cooker were found to be lower compared to the fresh sample. The conventional and microwave heating methods can be recommended as heat treatments that better preserve the original content of beneficial substances in broccoli. More studies are needed to preserve the content of health-promoting nutrients in consumed broccoli, gain new insights, and optimize the way broccoli is cooked. Cooking techniques and times had significant effects on the bioactive components, antioxidant activities, polyphenol contents, mineral results, and protein values of broccoli. The bioactive properties of broccoli samples cooked using conventional heating and microwave heating were established to be higher than the results of both fresh broccoli and broccoli cooked using the other three cooking techniques. The phenolic constituents of broccoli samples cooked with conventional heating and microwave heating were higher when compared to the results of fresh broccoli and broccoli cooked using the other cooking techniques. Therefore, the highest protein in broccoli samples was determined to be in the broccoli sample cooked by means of boiling in a vacuum bag, followed by the sample cooked using conventional boiling, boiling in a steam cooker, microwave heating, conventional heating, and the fresh sample in descending order.

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References

1. Podsedek, A.; Sosnowska, D.; Redzynia, M.; Anders, B. Antioxidant capacity and content of *Brassica oleracea* dietary antioxidants. *Int. J. Food Sci. Technol.* **2006**, *41* (Suppl. 1), 49–58. [CrossRef]
2. Wagner, A.E.; Terschluesen, A.M.; Rimbach, G. Health promoting effects of brassica-derived phytochemicals: From chemopreventive and anti-inflammatory activities to epigenetic regulation. *Oxidative Med. Cell. Longev.* **2013**, *2013*, 964539. [CrossRef] [PubMed]

3. Ciancaleoni, S.; Chiarenza, G.L.; Raggi, L.; Branca, F.; Negri, V. Diversity characterisation of broccoli (*Brassica oleracea* L. var. *italica* Plenck) landraces for their on-farm (in situ) safeguard and use in breeding programs. *Genet. Res. Crop Evol.* **2014**, *61*, 451–464. [CrossRef]
4. Thapa, U.; Prasad, P.H.; Rai, R. Studies on growth, yield and quality of broccoli (*Brassica oleracea italica* Plenck) as influenced by boron and molybdenum. *J. Plant Nutr.* **2016**, *39*, 261–267. [CrossRef]
5. Özer, Ç. Influence of different cooking methods on bioactıve properties of broccoli. *Int. J. Contemp. Tour. Res.* **2016**, *1*, 69–76. [CrossRef]
6. Moreno, D.A.; Carvajal, M.; Lopez-Berenguer, C.; García-Viguera, C. Chemical and biological characterisation of nutraceutical compounds of broccoli. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1508–1522. [CrossRef] [PubMed]
7. Mukherjee, V.; Mishra, P.K. Broccoli-an underexploited neutraceutical. *Sci. Res. Report.* **2012**, *2*, 291–294.
8. Ares, A.M.; Nozal, M.J.; Bernal, J. Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds. *J.Chromatograp. A* **2013**, *1313*, 78–95. [CrossRef]
9. Chen, A.Y.; Chen, Y.C. A Review of the Dietary Flavonoid, Kaempferol on Human Health and Cancer Chemoprevention. *Food Chem.* **2013**, *138*, 2099–2107. [CrossRef]
10. Miekus, N.; Marszałek, K.; Podlacha, M.; Iqbal, A.; Puchalski, C.; Swiergiel, A.H. health benefits of plant-derived sulfur compounds, Glucosinolates, and Organosulfur Compounds. *Molecules* **2020**, *25*, 3804. [CrossRef]
11. Miglio, C.; Chiavaro, E.; Visconti, A.; Fogliano, V.; Pellegrini, N. Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. *J. Agric. Food Chem.* **2008**, *56*, 139–147. [CrossRef] [PubMed]
12. Pellegrini, N.; Chiavaro, E.; Gardana, C.; Mazzeo, T.; Contino, D.; Gallo, M.; Riso, P.; Fogliano, V.; Porrini, M. Effect of different cooking methods on color, phytochemical concentration, and antioxidant capacity of raw and frozen *Brassica* vegetables. *J. Agric. Food Chem.* **2010**, *58*, 4310–4321. [CrossRef] [PubMed]
13. Tomás-Barberán, F.A.; Selma, M.V.; Espín, J.C. Interactions of gut microbiota with dietary polyphenols and consequences to human health. Current Opinion in Clin. *Nutr. Metab. Care* **2016**, *19*, 471–476. [CrossRef] [PubMed]
14. Ng, Z.X.; Chai, J.W.; Kuppusamy, U.R. Customized cooking method improves total antioxidant activity in selected vegetables. *Int. J. Food Sci. Nutr.* **2011**, *62*, 158–163. [CrossRef] [PubMed]
15. Diamante, M.S.; Vanz Borges, C.; Minatel, I.O.; Jacomino, A.P.; Basílio, L.S.P.; Monteiro, G.C.; Corrêa, C.R.; de Oliveira, R.A.; Pace Pereira Lima, G. Domestic cooking practices influence the carotenoid and tocopherol content in colored cauliflower. *Food Chem.* **2021**, *340*, 127901. [CrossRef] [PubMed]
16. Amakura, Y.; Umino, Y.; Tsuji, S.; Tonogai, Y. Influence of jam processing on the radical scavenging activity and phenolic content in berries. *J. Agric. Food Chem.* **2001**, *48*, 6292–6297. [CrossRef] [PubMed]
17. Ghafoor, K.; Al Juhaimi, F.; Özcan, M.M.; Uslu, N.; Ahmed, I.A.M.; Babiker, E.E. The effect of boiling, germination and roasting on bioactive properties, phenolic compounds, fatty acids and minerals of chia seed (*Salvia hispanica* L.) and oils. *Int. J. Gastron. Food Sci.* **2022**, *27*, 100447. [CrossRef]
18. Vallejo, F.; Tomas-Barberan, F.A.; Garcia-Viguera, C. Phenolic compound contents in edible parts of broccoli inflorescences after domestic cooking. *J. Sci. Food Agric.* **2003**, *83*, 1511–1516. [CrossRef]
19. Price, K.R.; Bacon, J.R.; Rhodes, M.J.C. Effect of storage and domestic processing on the content and composition of flavonol glucosides in onion (*Allium cepa*). *J. Agric. Food Chem.* **1997**, *45*, 938–942. [CrossRef]
20. Vallejo, F.; Tomas-Barberan, F.A.; Garcia-Viguera, C. Glucosinolates and vitamin C content in edible parts of broccoli inflorescences after domestic cooking. *Eur. Food Res. Technol.* **2002**, *215*, 310–316.
21. Ramirez, D.; Abellán-Victorio, A.; Beretta, V.; Camargo, A.; Moreno, D.A. Functional Ingredients from *Brassicaceae* Species: Overview and Perspectives. *Int. J. Mol. Sci.* **2020**, *21*, 1998. [CrossRef]
22. Do, T.N.; Hwang, E. Effects of different cooking methods on bioactive compound content and antioxidant activity of water spinach (*Ipomoea aquatica*). *Food Sci. Biotechnol.* **2015**, *24*, 799–806.
23. Zhan, L.; Pang, L.; Ma, Y.; Zhang, C. Thermal processing affecting phytochemical contents and total antioxidant capacity in broccoli (*Brassica oleracea* L.). *J. Food Process. Preserv.* **2017**, *42*, e13548. [CrossRef]
24. Zhao, C.; Liu, Y.; Lai, S.; Cao, H.; Guan, Y.; San Cheang, W.; Liu, B.; Zhao, K.; Miao, S.; Riviere, C.; et al. Effects of domestic cooking process on the chemical and biological properties of dietary phytochemicals. *Trends Food Sci. Technol.* **2019**, *85*, 55–66. [CrossRef]
25. Putriani, N.; Perdana, J.; Meiliana; Nugrahedi, P.Y. Effect of thermal processing on key phytochemical compounds in green leafy vegetables: A Review. *Food Rev. Int.* **2020**, *2020*, 783–811. [CrossRef]
26. Puupponen-Pimia, R.; Häkkinen, S.T.; Aarni, M.; Suortti, T.; Lampi, A.-M.; Eurola, M.; Piironen, V.; Nuutila, A.M.; Oksman-Caldentey, K.-M. Blanching and long-term freezing affect various bioactive compounds of vegetables in different ways. *J. Sci. Food Agric.* **2003**, *83*, 1389–1402. [CrossRef]
27. Williams, D.J.; Edwards, D.; Hamernig, I.; Jian, L.; James, A.P.; Johnson, S.K.; Tapsell, L.C. Vegetables containing phytochemicals with potential anti-obesity properties: A Review. *Food Res. Int.* **2013**, *52*, 323–333. [CrossRef]
28. Perdana, J.; den Besten, H.M.W.; Aryani, D.C.; Kutahya, O.; Fox, M.B.; Kleerebezem, M.; Boom, R.M.; Schutyser, M.A.I. Inactivation of *Lactobacillus plantarum* WCFS1 during Spray Drying and Storage Assessed with Complementary Viability Determination Methods. *Food Res. Int.* **2014**, *64*, 212–217. [CrossRef] [PubMed]

29. Liu, R.H. Potential synergy of phytochemicals in cancer prevention:mechanism of action. *J. Nutr.* **2004**, *134*, 3479S–3485S. [CrossRef]
30. Şat, İ.G.; Öz, Ö. Haşlama ve Kurutmanın Bazı Sebzelerin Bileşimi Üzerine Etkisi. *Adıyaman Üniv. Mühendis. Bilim. Derg.* **2015**, *3*, 54–62.
31. Nartea, A.; Fanesi, B.; Giardinieri, A.; Campmajó, G.; Lucci, P.; Saurina, J.; Pacetti, D.; Fiorini, D.; Frega, N.G.; Núñez, O. Glucosinolates and Polyphenols of Colored Cauliflower as Chemical Discriminants Based on Cooking Procedures. *Foods* **2022**, *11*, 3041. [CrossRef]
32. Cemeroğlu, B. *Meyve ve Sebze İşleme Teknolojisi*, 5th ed.; Nobel Akademik Yayıncılık: Ankara, Turkey, 2011; s.728.
33. Karabayır, C. *Kurutulmuş Sebzeler*; İhracatı Geliştirme Etüd Merkezi (İGEME): Ankara, Turkey, 2007.
34. dos Reis, L.C.R.; de Oliveira, V.R.; Hagen, M.E.K.; Jablonski, A.; Flôres, S.H.; de Oliveira Rios, A. Carotenoids, flavonoids, chlorophylls, phenolic compounds and antioxidant activity in fresh and cooked broccoli (*Brassica oleracea* var. *avenger*) and cauliflower *Brassica oleracea* var. *alphina* F1). *Food Sci. Technol.* **2015**, *63*, 177–183. [CrossRef]
35. Palermo, M.; Pellegrini, N.; Fogliano, V. The effect of cooking on the phytochemical content of vegetables. *J. Sci. Food Agric.* **2014**, *94*, 1057–1070. [CrossRef] [PubMed]
36. Amarowicz, R. Antioxidant activity of Maillard reaction products. *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 109–111. [CrossRef]
37. Liu, H.M.; Han, Y.F.; Wang, N.N.; Zheng, Y.Z.; Wang, X.D. Formation and antioxidant activity of maillard reaction products derived from different sugar-amino acid aqueous model systems of sesame roasting. *J. Oleo Sci.* **2020**, *69*, 391–401. [CrossRef] [PubMed]
38. Mansour, A.A.; Elshimy, N.M.; Shekib, L.A.; Sharara, M.S. Effect of domestic processing methods on the chemical composition and organoleptic properties of Broccoli and Cauliflower. *Am. J. Food Nutr.* **2015**, *3*, 125–130.
39. Gawlik-Dziki, U.; Jeżyna, M.; Świeca, M.; Dziki, D.; Baraniak, B.; Czyż, J. Effect of bioaccessibility of phenolic compounds on in vitro anticancer activity of broccoli sprouts. *Food Res. Int.* **2012**, *49*, 469–476. [CrossRef]
40. López-Hernández, A.A.; Ortega-Villarreal, A.S.; Rodríguez, J.A.V.; Lomelí, M.L.C.; González-Martínez, B.E. Application of different cooking methods to improve nutritional quality of broccoli (*Brassica oleracea* var. *italica*) regarding its compounds content with antioxidant activity. *Int. J. Gastron. Food Sci.* **2022**, *28*, 100510. [CrossRef]
41. Lafarga, T.; Bobo, G.; Viñas, I.; Zudaire, L.; Simó, J.; Aguiló-Aguayo, I. Steaming and sous-vide: Effects on antioxidant activity, vitamin C, and total phenolic content of Brassica vegetables. *Int. J. Gastron. Food Sci.* **2018**, *13*, 134–139. [CrossRef]
42. Turkmen, N.; Sari, F.; Velioglu, Y. The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chem.* **2005**, *93*, 713–718. [CrossRef]
43. Dolinsky, M.; Agostinho, C.; Ribeiro, D.; Rocha, G.D.S.; Barroso, S.G.; Ferreira, D. Effect of different cooking methods on the polyphenol concentration and antioxidant capacity of selected vegetables. *J. Culin. Sci. Technol.* **2016**, *14*, 1–12. [CrossRef]
44. Drabińska, N.; Ciska, E.; Szmatowicz, B.; Krupa-Kozak, U. Broccoli by-products improve the nutraceutical potential of gluten-free mini sponge cakes. *Food Chem.* **2018**, *267*, 170–177. [CrossRef] [PubMed]
45. Borja-Martínez, M.; Lozano-Sánchez, J.; Borrás-Linares, I.; Pedreño, M.A.; Sabater-Jara, A.B. Revalorization of broccoli by-products for cosmetic uses using supercritical fluid extraction. *Antioxidants* **2020**, *9*, 1195. [CrossRef]
46. Gunathilake, K.D.P.P.; Ranaweera, K.K.D.S.; Rupasinghe, H.P.V. Effect of different cooking methods on polyphenols, carotenoids and antioxidant activities of selected edible leaves. *Antioxidants* **2018**, *7*, 117. [CrossRef] [PubMed]
47. Liu, M.; Zhang, L.; Ser, S.L.; Cumming, J.R.; Ku, K.-M. Comparative phytonutrient analysis of broccoli by-products: The potentials for broccoli by-product utilization. *Molecules* **2018**, *23*, 900. [CrossRef] [PubMed]
48. Yilmaz, M.S.; Sakiyan, Ö.; Mazi, I.B.; Mazi, B.G. Phenolic content and some physical properties of dried broccoli as affected by drying method. *Food Sci. Technol. Int.* **2018**, *25*, 76–88. [CrossRef]
49. García, S.L.R.; Raghavan, V. Microwave-Assisted extraction of phenolic compounds from Broccoli (*Brassica oleracea*) stems, leaves, and florets: Optimization, characterization, and comparison with maceration extraction. *Recent Progress Nutr.* **2022**, *1*, 1–23.
50. Farnham, M.W.; Grusak, M.A.; Wang, M. Calcium and magnesium concentration of in bred and hybrid broccoli heads. *J. Am. Soc. Hort. Sci.* **2000**, *125*, 344–349. [CrossRef]
51. AOAC. *Official Methods of Analysis of Association of Official Analytical Chemists International*, 18th ed.; Horwitz, W., Ed.; AOAC Press: Arlington, VA, USA, 2005.
52. Girgin, N.; El, S.N. Effects of cooking on in vitro sinigrin bioaccessibility, total phenols, antioxidant and antimutagenic activity of cauliflower (*Brassica oleraceae* L. var. *Botrytis*). *J. Food Comp. Anal.* **2015**, *37*, 119–127. [CrossRef]
53. Yoo, K.M.; Lee, K.W.; Park, J.B.; Lee, H.J.; Hwang, I.K. Variation in major antioxidants and total antioxidant activity of Yuzu (*Citrus junos Sieb ex Tanaka*) during maturation and between cultivars. *J. Agric. Food Chem.* **2004**, *52*, 5907–5913. [CrossRef]
54. Hogan, S.; Zhang, L.; Li, J.; Zoecklein, B.; Zhou, K. Antioxidant properties and bioactive components of Norton (*Vitis aestivalis*) and Cabernet Franc (*Vitis vinifera*) wine grapes. *LWT-Food Sci. Technol.* **2009**, *42*, 1269–1274. [CrossRef]
55. Lee, S.K.; Mbwambo, Z.H.; Chung, H.S.; Luyengi, L.; Games, E.J.C.; Mehta, R.G. Evaluation of the antioxidant potential of natural products. *Comb. Chem. High Throughput Screen.* **1998**, *1*, 35–46. [CrossRef] [PubMed]
56. Skujins, S. *Handbook for ICP-AES (Varıan-Vista)*; A short guide to vista series ICP-AES operation; Varian Int: Zug, Switzerland, 1998.

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Phytochemical Characterization and Bioactivity Evaluation of Extracts Obtained via Ultrasound-Assisted Extraction of Medicinal Plant *Phedimus aizoon*

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Abstract: *Phedimus aizoon* has been utilized as a medicinal plant in Asia. However, the production of phytochemical-rich extracts from *P. aizoon* and the evaluation of their bioactivity are limited. Herein, phytochemical-rich extracts were prepared by ultrasound-assisted extraction of *P. aizoon*, with a high extraction yield of 16.56%. The extracts contained about 126 mg of phenolics and 31 mg of flavonoids per g of the extracts. The chromatographic analysis (GC-MS and HPLC analyses) identified 19 notable phytochemicals of the extracts from *P. aizoon*, including pentacosane, hexadecanoic acid, gallic acid, vanillic acid, and quercetin. The gallic acid content of the extracts was relatively high at 2.75 mg/g. The identified compounds are known to have various bioactivities, such as antioxidant, antibacterial, and antifungal activities. In fact, the prepared extracts exhibited antioxidant activity at 24–28% of that of ascorbic acid. In addition, it showed antibacterial activity against both *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria). This study highlights that *P. aizoon* deserves attention as a natural bioactive substance and emphasizes the need for applications of the extracts from *P. aizoon*.

Keywords: medicinal plant; *Phedimus aizoon*; bioactive compound; chromatographic analysis; gallic acid; vanillic acid

1. Introduction

Phedimus aizoon (formerly known as *Sedum aizoon*) is a perennial herbaceous species of the Crassulaceae family and is widely distributed in China, Korea, Japan, and Mongolia [1]. *P. aizoon* has been recognized as a medicinal and edible plant with various pharmacological effects, such as protecting the cardiovascular system, enhancing immunity, stopping bleeding, and tranquilizing the mind [2,3]. The pharmacological effects of *P. aizoon* (or *S. aizoon*) were introduced in ancient medical classics in China [3]. *P. aizoon* has been reported to contain a variety of bioactive constituents, such as flavonoids, phenolic acids, and triterpenes, which have been considered to contribute to its bioactivities [4,5]. Nevertheless, compared to other medicinal plants, there are few reports on the phytochemical characteristics and bioactivities of *P. aizoon*. For the sustainable utilization of *P. aizoon*, efficient extraction, isolation, identification, and activity evaluation of bioactive compounds are needed.

As one of the strategies for recovering bioactive compounds from plant material, the extraction process (techniques and conditions) can be optimized to maximize the extraction

yield of the target bioactive compound [6,7]. Prior to this, the extracts must be systematically characterized in terms of the aspects of their phytochemical compositions and bioactivities [8]. In previous studies, non-conventional techniques, such as ultrasound-assisted extraction (UAE) [9] and microwave-assisted extraction (MAE) [10], have been recommended as efficient methods to extract bioactive compounds including flavonoids from *P. aizoon*. However, the information on the phytochemical profiles and bioactivity of *P. aizoon* extracts obtained by using non-conventional techniques is limited. Previous studies detected bioactive compounds of *P. aizoon* extracts obtained by using a conventional extraction technique known as maceration, using various analyses such as chromatography, infrared spectroscopy, mass spectrometry, and nuclear magnetic resonance, and the following compounds were identified: caffeic acid, gallic acid, vanillic acid, luteolin, quercetin, kaempferol, myricetin, and rutin [3]. In addition, Odeh et al. [11] reported the presence of caffeic acid, catechin, gallic acid, hesperidin, and rutin in *S. rubens*, another species of *Sedum*. These phenolics and flavonoids contribute to the bioactivities and medicinal functions of *P. aizoon*; thus, their contents should be studied after the extraction process.

Herein, we aimed to perform the phytochemical characterization and bioactivity evaluation of *P. aizoon* extracts obtained by UAE. Total phenol and flavonoid contents were quantified, and the phytochemicals were analyzed by gas chromatography–mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) analyses. In addition, the bioactivities (antioxidant and antibacterial) of *P. aizoon* extracts were evaluated. The present study aims to contribute to advancing our biochemical knowledge of the medicinal plant *P. aizoon* and, in particular, to explore the potential of its extracts, which are rich in bioactive compounds, from the UAE process.

2. Materials and Methods

2.1. Chemicals

Ascorbic acid (or vitamin C), Folin–Ciocalteu reagent, $NaNO_2$, 2,2′-azino-bis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), Na_2CO_3, gallic acid, vanillic acid, luteolin, quercetin, catechin, rutin, and hesperidin were acquired from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol and myricetin were acquired from Tokyo Chemical Industry (Tokyo, Japan). Methanol, $AlCl_3$, and NaOH were acquired from Duksan Chemical (Ansan, Republic of Korea).

2.2. Plant Material

Phedimus aizoon (L.) ‘t Hart was collected in Tsagaannuur district, Huvsugul province, Mongolia, and identified by Dr. Badamtsetseg Bazarragchaa at the National History Museum of Mongolia in August 2015 (Figure 1). A voucher specimen (accession number KRIB 0070462) of the retained material is preserved at the herbarium of KRIBB.

2.3. Ultrasound-Assisted Extraction (UAE) of Bioactive Compounds from Phedimus aizoon

A mixture of leaves, shoots, and flowers of *P. aizoon* was used for the extraction. The plant materials were dried in the shade and powdered (water content: 19.1%). In general, methanol is commonly used in the extraction process of bioactive compounds, including flavonoids; thus, methanol was used as an extraction solvent [10,12]. A total of 55 g of plant material was immersed in 1 L of methanol (99.9%) and extracted using an ultrasonic extractor (SDN-900H, SD-ULTRASONIC, Co., Ltd., Seoul, Republic of Korea) for 30 cycles (40 KHz; 1500 W; 15 min sonication and 120 min standing) at room temperature. After extraction, the extracts were filtered and then concentrated at 45 °C using a rotary evaporator (N-1000SWD, EYELA, Tokyo, Japan) and freeze-dried.



Figure 1. Mongolian plant *Phedimus aizoon* (L.) 't Hart.

2.4. Phytochemical Analysis

2.4.1. Estimation of Phenolic Content and Flavonoid Content

The total phenolic and flavonoid contents of the extracts from *P. aizoon* were measured according to our previous study [12]. For the estimation of total phenolic content, 10 µL of the sample (i.e., the extracts dissolved in methanol), 790 µL of water, and 50 µL of Folin–Ciocalteu reagent were mixed and incubated at 30 °C. After 8 min, 150 µL of Na_2CO_3 (20% solution) was added and incubated at 25 °C. After 60 min, the absorbance (*A*) of the final mixture was recorded at 700 nm. The data are shown as mg caffeic acid equivalent (CAE) per g extracts. For the estimation of total flavonoid content, 50 µL of the sample and 30 µL of $NaNO_2$ (5% solution) were mixed and incubated at 25 °C. After 6 min, 50 µL of $AlCl_3$ (10% solution) was added and incubated at 25 °C. After 5 min, 300 µL of NaOH (1 M solution) and 1000 µL of water were added and incubated at 25 °C. After 15 min, the absorbance (*A*) of the final mixture was recorded at 510 nm. The data are shown as mg catechin equivalent (CE) per g extracts.

2.4.2. GC-MS Analysis

GC–MS analysis was performed according to previous studies [13,14] with minor modifications. The extracts from *P. aizoon* were dissolved in methanol, with a final concentration of about 5 µg/mL. The analysis was performed using GC 7890A, 5975C (Agilent, Santa Clara, CA, USA), equipped with a DB 5MS column (0.25 mm × 0.25 µm, 30 m length) with a single quadrupole detection system and an injection volume of 1 µL. The detailed conditions were set as follows: run time, 34 min; oven temperature, initially 60 °C and increased to 280 °C at a rate of 10 °C/min. The National Institute of Standards and Technology (NIST) Library database was used to identify compounds in *P. aizoon* extracts.

2.4.3. HPLC Analysis

For the quantification of several phytochemicals in the sample (i.e., the extracts dissolved in methanol), the HPLC–diode array detector (DAD) (G7117C, Agilent, Santa Clara,

CA, USA) analysis was conducted according to our previous study [12]. An INNO column C18 (5 μm, 4.6 mm × 250 mm) was used for analysis and the column temperature was set to 25 °C. A volume of 5 μL of the sample was injected, and the gradient mode was set as follows (solvent A, 0.03% phosphoric acid in deionized water; solvent B, acetonitrile): 0 min, 90% A; 15 min, 80% A; 28 min, 60% A; 36 min, 25% A; 38 min, 90% A; and 50 min, 90% A. The monitoring wavelengths were 250 nm (for vanillic acid), 280 nm (for caffeic acid and gallic acid), and 360 nm (for luteolin, quercetin, and kaempferol). The standard materials of the phytochemicals were selected based on a previous study, which reported the major phytochemicals of *P. aizoon* [3].

2.5. Assessment of Antioxidant and Antibacterial Activity

2.5.1. Antioxidant Activity

Various concentrations of the extracts (20–60 and 240–280 μg/mL) or ascorbic acid (7–11 and 50–90 μg/mL) were prepared and then used to evaluate their antioxidant activity. The ABTS method can be used to evaluate the antioxidant activity of hydrophilic and lipophilic compounds, whereas the DPPH method can only evaluate hydrophobic compounds. Therefore, the antioxidant activity of the extracts was assessed based on two methods [12,15]. After the ABTS cation radical scavenging reaction of *P. aizoon* extracts, the absorbance (*A*) was recorded at 734 nm. The radical scavenging activity was calculated using Formula (1). After the DPPH free radical scavenging reaction of *P. aizoon* extracts, the absorbance (*A*) was recorded at 517 nm. The radical scavenging activity was calculated using Formula (1).

$$\text{Radical scavenging activity (\%)} = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100, \quad (1)$$

where *A* is the absorbance at 734 nm or 517 nm (blank solution: methanol). The data are expressed as the concentration of *P. aizoon* extracts required to scavenge 50% of the initial radicals (i.e., IC_{50}).

2.5.2. Antibacterial Activity

The antibacterial activity of *P. aizoon* extracts was evaluated according to Dutta et al. [16] with minor modifications. *Escherichia coli* and *Staphylococcus aureus* were cultured in a nutrient broth medium for 24 h. The cultured broth with an optical density at 600 nm ($OD_{600\,nm}$) = 0.1 was inoculated into 5 mL of the nutrient broth medium. *P. aizoon* extracts were not included in the control group, and the extracts were added to the experimental group (final concentration of extracts in medium = 1 g/L). The broth was incubated at 37 °C and 180 rpm for 1 day, and the $OD_{600\,nm}$ of the broth cultured for 4, 8, and 24 h was measured. In addition, to confirm viable cells, the broth cultured for 24 h was spread on a nutrient agar plate and incubated at 37 °C for 1 day.

2.6. Statistical Analysis

The extraction process was performed in triplicate to determine the extraction yield from *P. aizoon*. Phenol and flavonoid content determination and bioactivity (antioxidant and antibacterial activity) evaluation were performed in triplicate. All data are expressed as the mean ± standard deviation. Chromatographic analysis was performed once. Significant differences in antioxidant and antibacterial activities between the control (or reference group) and the experimental group were identified by a *t*-test with Sigmaplot (version 12.5; Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Analysis of Phytochemicals in Extracts from Phedimus aizoon

Ultrasound-assisted extraction was performed to produce extracts from *P. aizoon*. As a result, the extraction yield was determined to be 16.56 ± 0.39% (dry weight basis) (Table 1). The content of phenolics and flavonoids in the extracts plays an important role in their

bioactivity, and thus, the analysis was carried out on *P. aizoon* extracts. The total phenolic content and the total flavonoid content of the extracts from *P. aizoon* were determined to be 126.3 ± 2.2 mg caffeic acid equivalent (CAE)/g extracts and 31.0 ± 0.5 mg catechin equivalent (CE)/g extracts, respectively, which means that about 12.6% and 3.1% of the extracts were phenolics and flavonoids (Table 1). Considering the extraction yield (16.56%) and the phenol and flavonoid content in the extracts, the yield of phytochemicals recovered from 100 g of *P. aizoon* was estimated to be about 2.09 g phenol and 0.51 g flavonoid/100 g biomass (dry weight basis).

Table 1. The extraction yield for *Phedimus aizoon* in the ultrasound-assisted extraction process and the contents of bioactive compounds in the extracts.

Extraction Yield	Total Phenol Content	Total Flavonoid Content
16.56 ± 0.39%	126.3 ± 2.2 mg CAE/g extracts	31.0 ± 0.5 mg CE/g extracts

CAE, caffeic acid equivalent; CE, catechin equivalent.

3.1.1. GC-MS Analysis

GC-MS analysis was performed to extend the phytochemical characterization of the extracts from *P. aizoon* to volatile compounds. As a result, the presence of at least 13 compounds was identified by searching in NIST Library (Table 2). Decane, undecane, dodecane, benzene, 1,3-bis(1,1-dimethylethyl)-, cyclohexasiloxane, dodecamethyl-, tetradecane, 2,4-di-tert-butylphenol, and hexadecanoic acid, methyl ester had a quality of at least 90%. The other five compounds (Dodecane, 1-iodo-, Cycloheptasiloxane, tetradecamethyl-, Pentacosane, 10-Methylnonadecane, and Hentriacontane) were identified as having a quality (library match) of at least 80%.

Table 2. Profiles of phytochemicals of extracts from *Phedimus aizoon* in GC-MS analysis.

No.	Compound [1]	RT (min)	Molecular Weight	Formula	Quality
1	Decane	6.290	142	$C_{10}H_{22}$	91
2	Undecane	7.886	156	$C_{11}H_{24}$	90
3	Dodecane	9.423	170	$C_{12}H_{26}$	94
4	Benzene, 1,3-bis(1,1-dimethylethyl)-	10.192	190	$C_{14}H_{22}$	95
5	Dodecane, 1-iodo-	10.522	296	$C_{12}H_{25}I$	80
6	Cyclohexasiloxane, dodecamethyl-	10.807	444	$C_{12}H_{36}O_6Si_6$	91
7	Tetradecane	12.242	198	$C_{14}H_{30}$	97
8	Cycloheptasiloxane, tetradecamethyl-	13.026	519	$C_{14}H_{42}O_7Si_7$	87
9	Pentacosane	13.377	352	$C_{25}H_{52}$	86
10	2,4-Di-tert-butylphenol	13.611	206	$C_{14}H_{22}O$	93
11	10-Methylnonadecane	13.948	282	$C_{20}H_{42}$	80
12	Hentriacontane	15.896	437	$C_{31}H_{64}$	86
13	Hexadecanoic acid, methyl ester	18.276	270	$C_{17}H_{34}O_2$	97

[1] Compounds with a quality of at least 80% are listed.

3.1.2. HPLC-DAD Analysis

Major bioactive compounds of *P. aizoon*, including phenolics and flavonoids, were analyzed by HPLC-DAD analysis (Figure A1). As known compounds of *Phedimus*, caffeic acid, gallic acid, vanillic acid, luteolin, quercetin, kaempferol, catechin, myricetin, rutin, and hesperidin were analyzed. Table 3 lists the detected compounds and their content in the extracts from *P. aizoon*. The contents (mg/g extracts) were high in the order of gallic acid (2.75), vanillic acid (0.50), quercetin (0.19), caffeic acid (0.13), luteolin (0.12), and kaempferol

(0.06). Other bioactive compounds such as catechin, myricetin, rutin, and hesperidin were not detected.

Table 3. Major phenolic acids and flavonoids in extracts from *Phedimus aizoon* in HPLC analysis.

Compound	Caffeic acid	Gallic acid	Vanillic acid	Luteolin	Quercetin	Kaempferol
Content (mg/g Extracts)	0.13	2.75	0.50	0.12	0.19	0.06

3.2. Bioactivities of Extracts from Phedimus aizoon

3.2.1. Antioxidant Activity

The antioxidant activity of *P. aizoon* extracts was evaluated based on radical scavenging activity. Two radicals (ABTS cation and DPPH free) were used for the evaluation, and the results are shown in Table 4. In an ABTS cation radical-based evaluation, 260.0 ± 2.6 μg/mL of the extracts was required for scavenging 50% of the radical. As a reference antioxidant, 72.9 ± 1.0 μg/mL of ascorbic acid was required for scavenging 50% of the ABTS cation radical. In a DPPH free radical-based evaluation, 41.0 ± 2.3 μg/mL of the extracts or 9.8 ± 0.2 μg/mL of ascorbic acid was required for scavenging 50% of the DPPH free radical. The results imply that the activity of the extracts to scavenge ABTS cation and DPPH free radicals is 28% and 24% of that of ascorbic acid, respectively.

Table 4. ABTS cation and DPPH free radical scavenging activity of *Phedimus aizoon* extracts. The asterisk indicates a significant difference compared to the reference antioxidant ($p < 0.05$).

Sample	**Radical Scavenging Activity (IC_{50}, μg/mL)**	
	ABTS Cation Radical	**DPPH Free Radical**
Phedimus aizoon extracts	260.0 ± 2.6 *	41.0 ± 2.3 *
Ascorbic acid (reference)	72.9 ± 1.0	9.8 ± 0.2

3.2.2. Antibacterial Activity

The antibacterial activity of the extracts from *P. aizoon* was evaluated based on cell growth profiling. The medium with 1 g/L of the extracts from *P. aizoon* was used for the qualitative evaluation, and the results are shown in Figure 2. In the medium without the extracts from *P. aizoon*, the growth of *E. coli* (Gram-negative bacteria) and *S. aureus* (Gram-positive bacteria) was increased by 24 h. The cell growth ($OD_{600\ nm}$) of *E. coli* and *S. aureus* measured at 4, 8, and 24 h was approximately 0.12, 0.29, and 0.71 and 0.12, 0.19, and 0.90, respectively. In contrast, no growth of *E. coli* and *S. aureus* cells was observed in the medium with the extracts from *P. aizoon* at 24 h. Preliminary investigations on the antibacterial activity of the positive control (ampicillin) showed that the minimum inhibitory concentration against *E. coli* and *S. aureus* was about 1.6 and 0.8 mg/L, respectively. Agar plates spread with 24 h-cultured broth of two strains proved that there were no viable cells of the two strains (Figure 2).

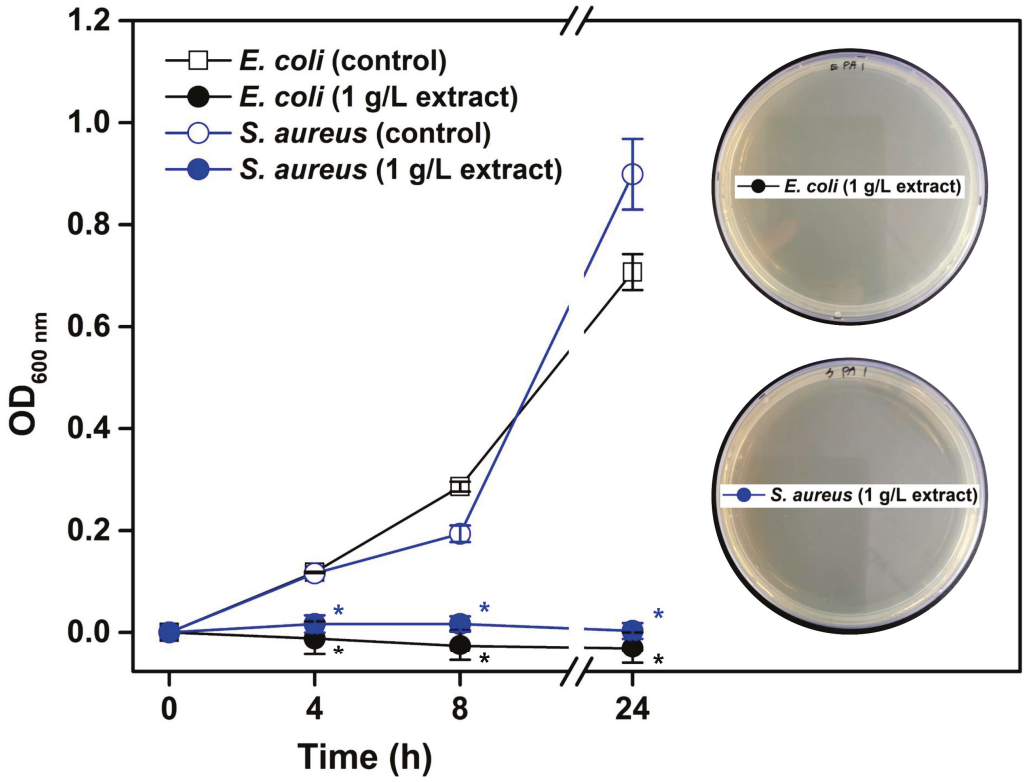


Figure 2. The profiles of the cell growth ($OD_{600\ nm}$) of *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria) in nutrient broth media with (experimental group) or without (control group) extracts from *Phedimus aizoon*. Viable cells of both bacteria were not observed in the nutrient agar medium after being spread with the 24 h-cultured broth and incubated, as shown in the right side. The asterisk indicates a significant difference compared to the control group ($p < 0.05$).

4. Discussion

The extraction yield in the ultrasound-assisted extraction (UAE) process for *P. aizoon* was about 16.56%, and the total phenol and flavonoid contents of the extracts were 126.3 ± 2.2 and 31.0 ± 0.5 mg/g, respectively (Table 1). This means that the phenol yield and flavonoid yield were 1.69% (i.e., 1692.4 mg/g) and 0.42% (i.e., 415.4 mg/g), respectively. Few previous studies have quantified the total phenol and flavonoid contents (or yields) of *P. aizoon* extracts. Among the conventional extraction techniques, in the maceration process for *P. aizoon*, phenol or flavonoid yield was not available. In the Soxhlet extraction process for *P. aizoon*, the flavonoid yield was determined to be approximately 18.67 mg/g [10]. In the case of non-conventional extraction processes, the flavonoid yield was achieved at approximately 24.87 mg/g under optimal MAE conditions (20 min, 57 °C, 20 mL solvent/g solid, and 80.6% ethanol) [10]. Yin and Batbatan [9] reported the optimal conditions of the UAE process for *P. aizoon*, and the flavonoid yield was 10.77 mg/g under the optimal conditions (1 g solid/55 mL solvent, 60% ethanol, 45 °C, 25 min, and 150 W ultrasound power). The relatively high flavonoid yield in the present study can be attributed to the sufficient time (3 days in total) of the UAE process (see Section 2.3). Further studies should propose optimized UAE conditions to obtain high yields in a shorter time.

The results of the GC-MS analysis identified various phytochemical and bioactive compounds in the extracts from *P. aizoon* (Table 2). Cyclohexasiloxane, dodecamethyl- has been reported to have hair-conditioning, antimicrobial, antiseptic, and skin-conditioning activities [17]. Cycloheptasiloxane, tetradecamethyl- also has various bioactivities such as antibacterial, immunomodulatory, antitumor, antifungal, and antifouling activities [17]. In addition, the biological activities of various phytochemicals detected by GC-MS analysis have been reported earlier: tetradecane, antioxidant and antifungal activities [18]; pentacosane, antibacterial activity [19]; 2,4-Di-tert-butylphenol, antioxidant, anti-inflammatory,

antibacterial, antiviral, and antifungal activities [20]; hexadecanoic acid, methyl ester, antioxidant and antibacterial activities [21]. As a result of the HPLC-DAD analysis, various phytochemical and bioactive compounds in the extracts from *P. aizoon* were quantified (Table 3). Overall, compounds that previous studies have identified as present in *P. aizoon* were detected. Lin et al. [22] identified eleven compounds of *P. aizoon* by IR, MS, and NMR, and caffeic acid, gallic acid, vallinic acid, luteolin, quercetin, and kaempferol were contained in the detected compounds. Xu et al. [23] also identified gallic acid, quercetin, and kaempferol in *P. aizoon* extracts using UPLC-DAD-Q-TOF-MS. Phenolic acids (caffeic acid, gallic acid, and vanillic acid) and flavonoids (luteolin, quercetin, and kaempferol) contribute to a variety of biological activities, including antioxidant, antibacterial, anti-inflammatory, and anti-cancer [24]. Thus, *P. aizoon* extracts containing these phenolics and flavonoids were expected to exhibit antioxidant and antibacterial activities.

In fact, *P. aizoon* extracts showed antioxidant and antibacterial activities (Table 4 and Figure 2). Qi et al. [25] optimized process conditions to maximize the flavonoid extraction yield from *P. aizoon* and evaluated the ABTS cation and DPPH free radical scavenging activities of the extracts based on their flavonoid concentration: (1) *P. aizoon* extracts and vitamin C (i.e., ascorbic acid) at 0.3 mg/mL scavenged about 96.2% and 99.0% of ABTS cation radicals, respectively; (2) *P. aizoon* extracts and vitamin C at 2.0 mg/mL scavenged about 85.9% and 98.8% of DPPH free radicals, respectively [25]. In this study, the IC_{50} of the prepared *P. aizoon* extracts was 260 μg/mL and 41 μg/mL for ABTS cation and DPPH free radicals, respectively (Table 4). Nevertheless, due to the limited number of previous studies evaluating the antioxidant activity of *P. aizoon* in vitro and the different criteria for the concentration (extract or flavonoid), the antioxidant properties of *P. aizoon* extracts as natural antioxidants should be further evaluated.

P. aizoon extracts showed antibacterial activity against both *E. coli* and *S. aureus* (Figure 2). Several previous studies have reported the antibacterial activity of *P. aizoon* extracts against the Gram-negative bacteria *Aeromonas* [26], *Shewanella putrefaciens* [27], and *Pseudomonas fragi* [5]. In contrast, Oyungerel and Kim [28] reported that *P. aizoon* extracts showed antibacterial activity against *S. aureus*, but not against *E. coli*. Based on these two previous studies and the current study, it is assumed that *P. aizoon* extracts have antibacterial activity against both Gram-negative and Gram-positive bacteria. The antimicrobial activity of *P. aizoon* extracts was summarized as the disruption of cellular metabolic processes, redox homeostasis, and the cause of damage to the bacterial surface and the membrane [3]. The treatment of Gram-negative bacteria *Pseudomonas fragi* with flavonoids from *P. aizoon* resulted in the disruption of the intracellular sulfate assimilation pathway and disturbances in glutathione redox homeostasis [5]. Therefore, the antibacterial activity of the extract prepared in this study against *E. coli* could be attributed to these mechanisms. Until now, regarding the antibacterial activity of *P. aizoon* extracts, advanced research (antibacterial mechanism, application as natural replacement for preservatives) has been focused on Gram-negative bacteria [5,26,27]; thus, further studies should be conducted in depth on Gram-positive bacteria. In addition, for industrial production of *P. aizoon* extracts, an evaluation of the usability of solvents that are generally recognized as safe (GRAS) and optimization of the extraction process can be performed [29]. The produced *P. aizoon* extracts are expected to be utilized as bioactive compounds for the fabrication of antioxidant and antibacterial polymers and nanocomposites [30,31].

5. Conclusions

In conclusion, this study established the composition and bioactivity of phytochemical-rich *P. aizoon* extracts with high potential as natural bioactive substances. By applying the ultrasound-assisted extraction (UAE) process, the extracts were produced from *P. aizoon* with a yield of about 16.56%, of which more than 12.6% were phenolic compounds. Well-known bioactive compounds such as cyclohexasiloxane, dodecamethyl-, tetradecane, pentacosane, and hexadecanoic acid were identified, and six compounds were quantified, including gallic acid, vanillic acid, and quercetin. Of these, gallic acid was present in the

highest amount at 2.75 mg/g extract. The prepared *P. aizoon* extracts showed outstanding ABTS cation radical scavenging activity (IC_{50}: 260.0 ± 2.6 μg/mL) and DPPH free radical scavenging activity (IC_{50}: 41.0 ± 2.3 μg/mL), which were 28% and 24% of the activity of ascorbic acid. Furthermore, the antibacterial activity of *P. aizoon* extracts was effective against both Gram-negative and Gram-positive bacteria. This study provides useful information for further phytochemical and biological studies on *P. aizoon* regarding the aspects of in vivo bioactivity, antibacterial mechanism, and applications.

Author Contributions: Conceptualization, J.L. and H.Y.Y.; methodology, M.K., H.S. and S.K.; validation, T.L. and H.Y.Y.; formal analysis, J.L. and M.K.; investigation, A.J., S.J. and S.-Y.K.; resources, A.J., S.J. and S.-Y.K.; writing—original draft preparation, J.L.; writing—review and editing, T.L. and H.Y.Y.; visualization, H.S. and S.K.; supervision, H.Y.Y.; project administration, T.L. and H.Y.Y.; funding acquisition, H.Y.Y. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article; further inquiries can be directed to the corresponding author.

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

Appendix A

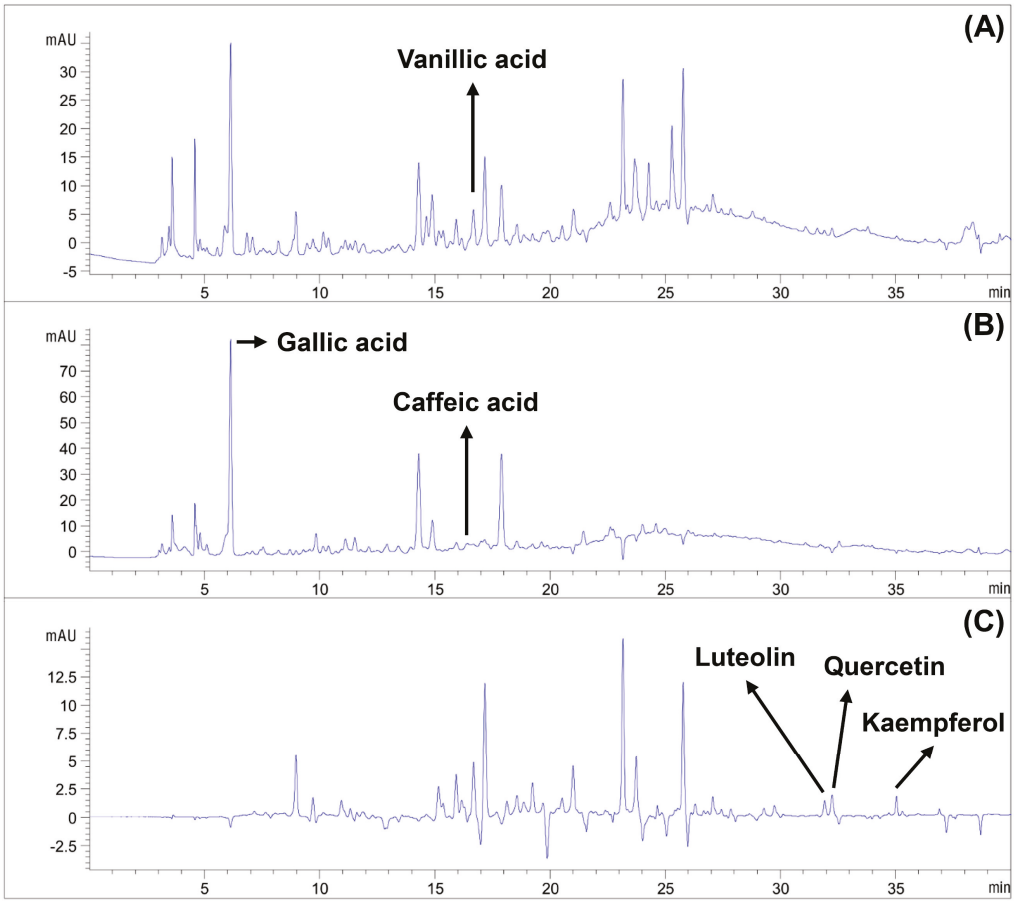


Figure A1. High-performance liquid chromatography chromatograms for *Phedimus aizoon* extracts. The detector monitored at wavelengths of 250 nm (**A**), 280 nm (**B**), and 360 nm (**C**).

References

1. Kim, Y.; Kim, S.H.; Yang, J.; Cho, M.S.; Koldaeva, M.; Ito, T.; Maki, M.; Kim, S.C. Plastome-based backbone phylogeny of East Asian *Phedimus* (*Subgenus Aizoon*: Crassulaceae), with special emphasis on Korean endemics. *Front. Plant Sci.* **2023**, *14*, 1089165. [CrossRef] [PubMed]
2. Liu, Y.; He, Z.; Xie, Y.; Su, L.; Zhang, R.; Wang, H.; Li, C.; Long, S. Drought resistance mechanisms of *Phedimus aizoon* L. *Sci. Rep.* **2021**, *11*, 13600. [CrossRef] [PubMed]
3. Wang, B.L.; Ge, Z.K.; Qiu, J.R.; Luan, S.Q.; Hao, X.C.; Zhao, Y.H. *Sedum aizoon* L.: A review of its history, traditional uses, nutritional value, botany, phytochemistry, pharmacology, toxicology, and quality control. *Front. Pharmacol.* **2024**, *15*, 1349032. [CrossRef] [PubMed]
4. Xu, T.; Wang, Z.; Lei, T.; Lv, C.; Wang, J.; Lu, J. New flavonoid glycosides from *Sedum aizoon* L. *Fitoterapia* **2015**, *101*, 125. [CrossRef] [PubMed]
5. Wang, H.; Xu, F.; Zhang, X.; Shao, X.; Wei, Y.; Wang, H. Transcriptomic analysis reveals antibacterial mechanism of flavonoids from *Sedum aizoon* L. against *Pseudomonas fragi*. *Food Control* **2021**, *134*, 108755. [CrossRef]
6. Jeon, H.-J.; Kim, J.-H. Effect of Gas Bubbles on the Recovery Efficiency of Paclitaxel from Biomass of *Taxus chinensis* in Ultrasonic Extraction. *Biotechnol. Bioprocess Eng.* **2023**, *28*, 545–553. [CrossRef]
7. Oh, H.; Kim, J.H. Development of an ultrasound-negative pressure cavitation fractional precipitation for the purification of (+)-dihydromyricetin from biomass. *Korean J. Chem. Eng.* **2023**, *40*, 1133–1140. [CrossRef]
8. Tran, T.K.; Ha, P.T.T.; Henry, R.J.; Nguyen, D.N.T.; Tuyen, P.T.; Liem, N.T. Polyphenol Contents, Gas Chromatography-Mass Spectrometry (GC–MS) and Antibacterial Activity of Methanol Extract and Fractions of *Sonneratia caseolaris* Fruits from Ben Tre Province in Vietnam. *J. Microbiol. Biotechnol.* **2024**, *34*, 94–102. [CrossRef] [PubMed]
9. Yin, Q.; Batbatan, C.G. Optimization of enzymatic-assisted ultrasonic extraction process of total flavonoids from *Sedum aizoon* L. and its antioxidant activity. In Proceedings of the 2023 International Conference on Food Science and Bio-Medicine, Guangzhou, China, 15–17 September 2023; BIO Web of Conferences; EDP Sciences: Les Ulis, France, 2023; p. 02004. [CrossRef]
10. Jin, C.; Wei, X.; Yang, S.; Yao, L.; Gong, G. Microwave-assisted Extraction and Antioxidant Activity of Flavonoids from *Sedum aizoon* Leaves. *Food Sci. Technol. Res.* **2017**, *23*, 111–118. [CrossRef]
11. Odeh, A.A.; Al-Jaber, H.I.; Barhoumi, L.M.; Al-Fawares, O.L.; Shakya, A.K.; Al-Qudah, M.A.; Al-Sanabra, O.M. Phytochemical and bioactivity evaluation of secondary metabolites and essential oils of *Sedum rubens* growing wild in Jordan. *Arab. J. Chem.* **2023**, *16*, 104712. [CrossRef]
12. Lee, J.; Song, Y.; Son, H.; Kim, S.; Lee, K.H.; Bazarragchaa, B.; Lee, C.; Yoo, H.Y. Phytochemical and Antioxidant Characterization of Extracts from Unexplored Medicinal Plants *Salix schwerinii* and *Salix kochiana*. *Horticulturae* **2023**, *9*, 955. [CrossRef]
13. Kusar, S.; Saddiqe, Z.; Ali, F.; Bashir, S.; Zubairi, T. GCMS and HPLC profiling, antioxidant and anti-inflammatory activities of *Crotalaria medicaginea* Lamk. *S. Afr. J. Bot.* **2024**, *168*, 196–208. [CrossRef]
14. Dinore, J.M.; Patil, H.S.; Farooqui, S.; Pradhan, V.; Farooqui, M. GC/MS and LC/MS phytochemical analysis of *Vigna unguiculata* L. Walp pod. *Chem. Biodivers.* **2023**, *20*, e202200048. [CrossRef]
15. Akhter, P.; Bhatti, T.Y.; Shafiq, I.; Jamil, F.; Nazar, R.; Nazir, M.S.; Hassan, S.U.; Hussain, M.; Park, Y. Antioxidant activity of sea buckthorn (*Hippophae rhamnoides*) seed oil extracted using various organic solvents. *Korean J. Chem. Eng.* **2023**, *40*, 2914–2920. [CrossRef]
16. Dutta, R.K.; Nenavathu, B.P.; Gangishetty, M.K.; Reddy, A.V.R. Studies on antibacterial activity of ZnO nanoparticles by ROS induced lipid peroxidation. *Colloids Surf. B Biointerfaces* **2012**, *94*, 143–150. [CrossRef]
17. Rasyid, A.; Putra, M.Y. Antibacterial and antioxidant activity of sea cucumber extracts collected from Lampung waters, Indonesia. *Kuwait J. Sci.* **2023**, *50*, 615–621. [CrossRef]
18. Khan, I.H.; Javaid, A. Antifungal, antibacterial and antioxidant components of ethyl acetate extract of quinoa stem. *Plant Prot.* **2019**, *3*, 125–130. [CrossRef]
19. Roopa, M.S.; Shubharani, R.; Rhetso, T.; Sivaram, V. Comparative analysis of phytochemical constituents, free radical scavenging activity and GC-MS analysis of leaf and flower extract of *Tithonia diversifolia* (Hemsl.) A. Gray. *Int. J. Pharm. Sci. Res.* **2020**, *11*, 5081–5090.
20. Zhao, F.; Wang, P.; Lucardi, R.D.; Su, Z.; Li, S. Natural Sources and Bioactivities of 2,4-Di-Tert-Butylphenol and Its Analogs. *Toxins* **2020**, *12*, 35. [CrossRef]
21. Ganesan, T.; Subban, M.; Britto, D.; Leslee, C.; Kuppannan, S.B.; Seedevj, P. Structural characterization of n-hexadecanoic acid 382 from the leaves of *Ipomoea eriocarpa* and its antioxidant and antibacterial activity. *Biomass Convers. Biorefin.* 2022, *early access*. [CrossRef]
22. Lin, Z.C.; Fang, Y.J.; Huang, A.Y.; Chen, L.Y.; Guo, S.H.; Chen, J.W. Chemical constituents from *Sedum aizoon* and their hemostatic activity. *Pharm. Biol.* **2014**, *52*, 1429–1434. [CrossRef]
23. Xu, T.; Lv, C.; Lu, J. The fingerprint and flavonoids contents by HPLC and the UPLC-DAD-Q-TOF-MS analysis of *Sedum aizoon* L. *J. Polyphen.* **2019**, *1*, 23–32.
24. Kim, S.; Lee, K.H.; Lee, J.; Lee, S.K.; Chun, Y.; Lee, J.H.; Yoo, H.Y. Efficient Recovery Strategy of Luteolin from Agricultural Waste Peanut Shells and Activity Evaluation of Its Functional Biomolecules. *Int. J. Mol. Sci.* **2023**, *24*, 12366. [CrossRef]
25. Qi, X.; Lu, X.T.; Sun, X.H.; Lin, C.Q.; Cui, C.B. The regulatory effect of total flavonoids of *Sedum aizoon* L. on oxidative stress in type 1 diabetic mice. *Curr. Res. Food Sci.* **2022**, *5*, 1140–1147. [CrossRef]

26. Xu, F.; Cao, S.; Wang, C.; Wang, K.; Wei, Y.; Shao, X.; Wang, H. Antimicrobial activity of flavonoids from *Sedum aizoon* L. against *Aeromonas* in culture medium and in frozen pork. *Food Sci. Nutr.* **2019**, *7*, 3224–3232. [CrossRef]
27. Wang, J.; Chi, Z.; Zhao, K.; Wang, H.; Zhang, X.; Xu, F.; Shao, X.; Wei, Y. A transcriptome analysis of the antibacterial mechanism of flavonoids from *Sedum aizoon* L. against *Shewanella putrefaciens*. *World J. Microbiol. Biotechnol.* **2020**, *36*, 94. [CrossRef]
28. Oyungerel, S.; Yim, J.H. Antimicrobial Activity of Some Mongolian Plants. *Mong. J. Biol. Sci.* **2023**, *21*, 3–13. [CrossRef]
29. Shin, H.; Lee, J.; Bae, J.; Lee, K.H.; Yoo, H.Y.; Park, C. Enhancement of dieckol extraction yield from *Ecklonia cava* through optimization of major variables in generally recognized as safe solvent-based process. *Front. Mar. Sci.* **2023**, *10*, 1287047. [CrossRef]
30. Ul-Islam, M.; Alhajaim, W.; Fatima, A.; Yasir, S.; Kamal, T.; Abbas, Y.; Khan, S.; Khan, A.H.; Manan, S.; Ullah, M.W.; et al. Development of low-cost bacterial cellulose-pomegranate peel extract-based antibacterial composite for potential biomedical applications. *Int. J. Biol. Macromol.* **2023**, *231*, 123269. [CrossRef]
31. Yousefinia, A.; Khodadadi, M.; Mortazavi-Derazkola, S. An efficient biosynthesis of novel ZnO/CuO nanocomposites using *berberis vulgaris* extract (ZnO/CuO@ *BV*ENCs) for enhanced photocatalytic degradation of pollution, antibacterial and antifungal activity. *Environ. Technol. Innov.* **2023**, *32*, 103340. [CrossRef]

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Effects of Gamma Irradiation on Changes in Chemical Composition and Antioxidant Activity of *Euphorbia maculata* Callus

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Abstract: In this study, we investigated the effects of gamma irradiation on the antioxidant activity and metabolite profiles of *Euphorbia maculata* calli (PC3012). Gamma irradiation at various doses (0, 0.05, 0.5, and 10 kGy) significantly enhanced the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^+$) radical scavenging activities of the callus extracts of PC3012 in a dose-dependent manner. High-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-Q-TOF/MS) analyses revealed that irradiation increased the lysophospholipid content, although no new antioxidant compounds were formed. Furthermore, a PLS-DA analysis revealed evident metabolic differences between non-irradiated and irradiated samples, which were further verified by statistical validation. These findings suggest that gamma irradiation induces specific biochemical modifications that enhance the bioactive properties of PC3012 calli. This technology exhibits potential for utilization in the natural product and food sectors, particularly in the development of functional foods and nutraceuticals with improved health benefits.

Keywords: *Euphorbia maculata*; gamma irradiation; plant-derived callus; radical scavenging; UPLC-QTOF/MS

1. Introduction

Studies were conducted approximately 30 years ago to enhance the production of secondary metabolites by inducing alterations in plant metabolic enzymes [1]. Several studies have demonstrated that irradiation under controlled conditions can result in beneficial hormonal responses in plants; therefore, exposure to low doses of radiation may have beneficial effects on plant growth [2]. Recent research is beginning to elucidate the molecular basis of these alterations, as well as their relationship with phenotypic changes. Heat shock proteins, the proteasome, activation of the kinase cascade, nitrogen metabolism, plant hormone balance, and antioxidant response mechanisms were all observed to undergo changes. It is possible that these alterations are the basis for hormonal responses in irradiated plants [3]. Gamma irradiation is a widely used technique for food preservation, sterilization, and the enhancement of plant-derived bioactive properties [4]. This process involves the exposure of substances to gamma rays, which can induce various biochemical

changes, potentially leading to improved biological activities [5]. However, investigations into the formation of natural materials using such radiation are inadequate, and studies on the activity of secondary metabolites in many different plants are required. Recent research has shown that gamma irradiation can significantly alter plant metabolism, particularly in the regulation of secondary metabolites such as chlorophylls, carotenoids, and anthocyanins. For instance, in *Arabidopsis thaliana*, gamma irradiation has been linked to delayed senescence and enhanced photosynthetic efficiency by preserving the integrity of chloroplasts and thylakoid membranes [6,7]. These findings suggest that gamma irradiation not only serves as a mutagenic agent but also as a potential enhancer of plant resilience and bioactive compound production, thereby highlighting the need for further studies on its effects across various plant species.

Euphorbia maculata, a member of the family Euphorbiaceae, is extensively naturalized in Korea, Japan, and China [8]. This plant is characterized by soft, prostrate, and branched stems that spread along the ground, and the leaves have distinctive red spots on the central vein. Traditionally, *E. maculata* has been used to alleviate bleeding and various ailments, including epistaxis, wounds, diarrhea, dysentery, carbuncles, and warts [9,10]. Previous phytochemical investigations of *E. maculata* have led to the isolation of several novel compounds, such as triterpenoids, flavonoids, tannins, and phenolic acids [11,12]. Pharmacological studies have confirmed its wide variety of biological activities, including antibacterial, antifungal, antioxidant, anti-inflammatory, anti-osteoporotic, anticancer, antiplatelet, anti-tyrosinase, and anti-obesity effects [13]. However, there have been no studies on the functionality and primary ingredients of calli.

Callus culture, the in vitro cultivation of plant cells, offers a controlled environment for studying and enhancing the production of valuable phytochemicals [14]. This technique is crucial for investigating the biosynthetic pathways and stress responses of plants, leading to the development of novel compounds with enhanced bioactivity. Callus cultures are extensively used in a variety of research disciplines, including plant physiology, biochemistry, and pharmacology, and are also employed in the production of medicinal compounds, genetic modifications, and the conservation of rare plant species [15]. In the present study, we investigated the effects of gamma irradiation on the antioxidant activity and metabolite profiles of *E. maculata* callus. By employing assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^+$) radical scavenging activities, along with an advanced ultra-performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-Q-TOF/MS) analysis, we aimed to elucidate the specific biochemical changes induced by gamma irradiation. This study aimed to contribute to the growing body of knowledge on the use of irradiation to enhance the functional properties of natural products, with potential applications in the development of functional foods and nutraceuticals.

2. Results

2.1. Radical Scavenging Ability of Plant-Derived Callus by Gamma Irradiation

Plant-derived callus cultures are valuable because of their ability to produce high concentrations of bioactive compounds [16]. In this study, we evaluated the radical scavenging activities of three plant-derived calli (*E. maculata*, *Punica granatum*, and *Potentilla fragarioides*) after gamma irradiation. Figure 1 shows that gamma irradiation significantly affects the antioxidant activities of the calli, as evidenced by changes in DPPH and $ABTS^+$ radical scavenging activities at a concentration of 100 μg/mL. The DPPH radical scavenging activity of *E. maculata* calli (PC0312) increased from 61.0% to 85.9%, whereas that of *P. fragarioides* calli (PC3062) slightly improved from 34.1% to 41.4%. Conversely, the DPPH radical scavenging activity of *P. granatum* calli (PC3026) decreased from 61.8% to 31.8% (Figure 1A). Similarly, the $ABTS^+$ radical scavenging activity of the PC3012 extract increased from 74.8% to 94.0% (Figure 1B). Among the three callus extracts, *E. maculata* callus (PC0312) exhibited the highest increase in both DPPH and $ABTS^+$ scavenging activities, indicating greater sensitivity to gamma irradiation.

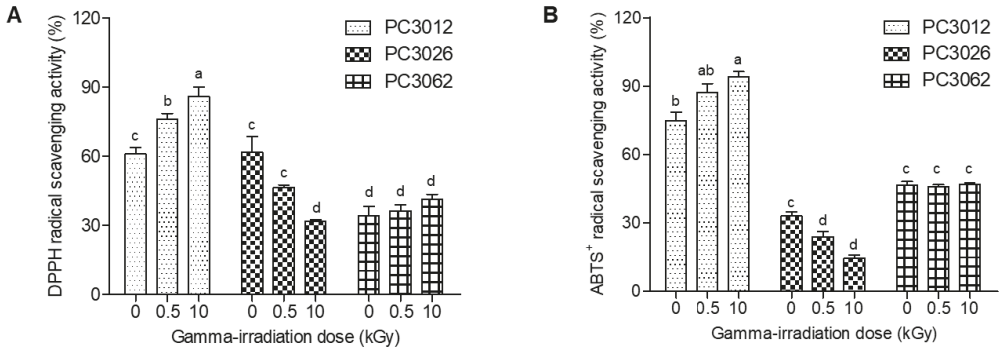


Figure 1. DPPH and ABTS$^+$ radical scavenging activities of three plant-derived calli after gamma irradiation. (**A**) DPPH radical scavenging assay; (**B**) ABTS$^+$ radical scavenging assay. Radical scavenging ability was measured at 100 μg/mL. The results are expressed as mean ± SD (n = 3). Statistical analyses were performed using a two-way ANOVA, followed by Tukey's post hoc test. Different letters represent statistically significant differences at $p < 0.05$. PC0312: *E. maculata* callus; PC3026: *P. granatum* callus; PC3062: *P. fragarioides* callus.

2.2. Radical Scavenging Ability of E. maculata Callus after Different Doses of Gamma Irradiation

E. maculata extracts have shown a diverse range of functional properties, including antioxidant, anti-inflammatory, and antimicrobial activities [17]. In our study, *E. maculata* extracts exhibited the greatest increase in antioxidant activity (Figure 1). However, research related to *E. maculata* calli is scarce. In this experiment, we evaluated the antioxidant activities of *E. maculata* calli (PC3012) exposed to various gamma irradiation doses. Figure 2 depicts the antioxidant activity of *E. maculata* calli (PC3012), at different gamma irradiation doses (0, 0.05, 0.5, and 10 kGy). The DPPH radical scavenging activity increased from an initial value of 61.0% to 70.9%, 76.2%, and 85.9% at 0.05, 0.5, and 10 kGy of gamma irradiation, respectively, showing a clear dose-dependent activity enhancement (Figure 2A). Similarly, the ABTS$^+$ radical scavenging activity increased from 74.8% at 0 kGy to 94.0% at 10 kGy, indicating a significant improvement in antioxidant capacity with increasing gamma irradiation doses (Figure 2B). To further understand the specific changes in antioxidant compounds, we hypothesized that gamma irradiation modifies key phytochemicals within the callus. Consequently, we performed HPLC and UPLC-Q-TOF/MS analyses to identify and characterize the specific substances that have changed after irradiation.

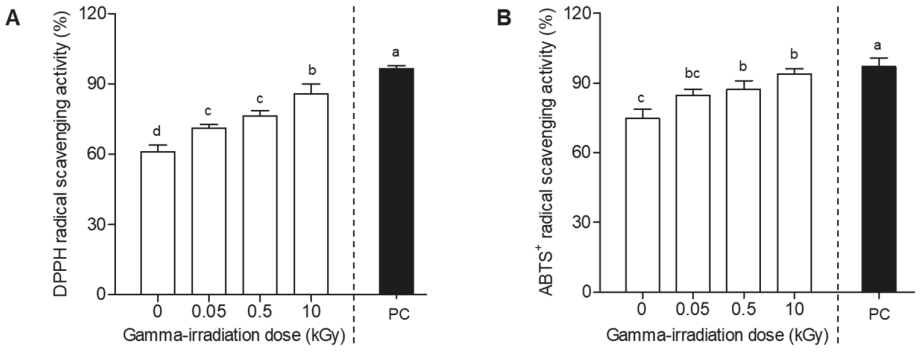


Figure 2. DPPH and ABTS$^+$ radical scavenging activities of *E. maculata* callus extracts at gamma radiation doses of 0, 0.05, 0.5, and 10 kGy. (**A**) DPPH radical scavenging assay; (**B**) ABTS$^+$ radical scavenging assay. Radical scavenging ability was measured at 100 μg/mL. The results are expressed as mean ± SD (n = 3). Statistical analyses were performed using a two-way ANOVA, followed by Tukey's post hoc test. Different letters represent statistically significant differences at $p < 0.05$. PC: (+)-Catechin used as a positive control.

2.3. High-Performance Liquid Chromatography (HPLC) Analysis of E. maculata Callus by Gamma Irradiation

A high-performance liquid chromatography (HPLC) analysis revealed significant differences in the chemical profiles of the non-irradiated and irradiated *E. maculata* callus extracts. The 10 kGy irradiated samples exhibited higher peak intensities and additional peaks at 254 nm (Figure 3A) and 360 nm (Figure 3B) than the non-irradiated and lower gamma-dose samples. No significant difference was observed between the calli extracts exposed to low irradiation doses (0.05 and 0.5 kGy) and the non-irradiated sample. The 10 kGy samples exhibited more pronounced peaks at 11.6 and 13.8 min retention times, indicating that higher irradiation doses contribute to an increase in specific compounds. These results suggest that the gamma irradiation induced changes in the metabolite profiles of the callus. The increased peak intensities and appearance of new peaks in the irradiated samples imply the enhanced production of certain phytochemicals and the formation of novel compounds. These changes enhanced the bioactive properties of the extracts.

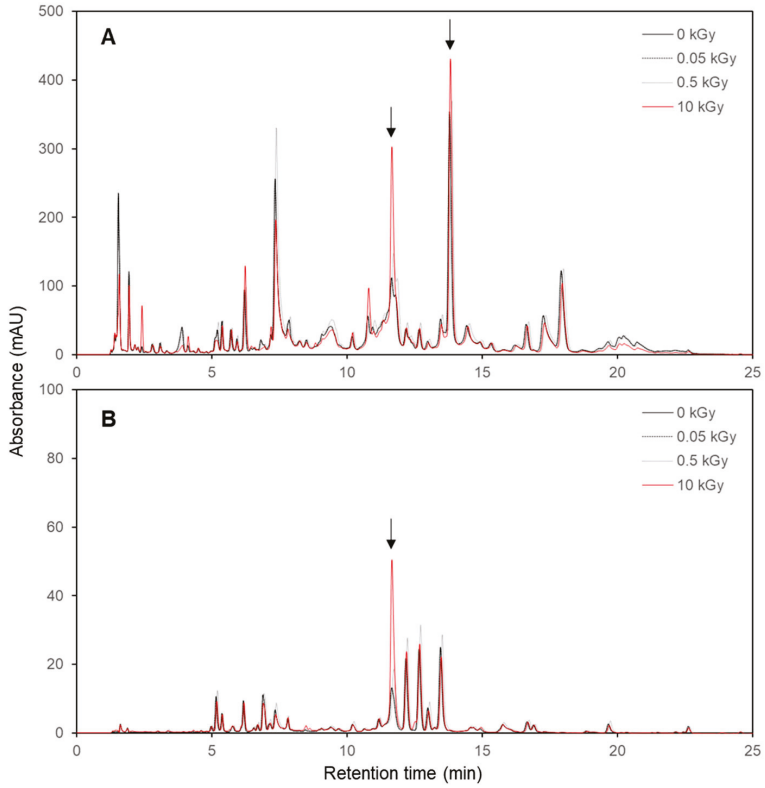


Figure 3. HPLC chromatograms of non-irradiated and irradiated *E. maculata* (PC3012) callus extracts. (**A**) 254 nm; (**B**) 360 nm. See the Materials and Methods section for experimental conditions. Arrow shows the increased peak by gamma irradiation in plant callus.

2.4. Identification of Compounds Produced in E. maculata Callus by Gamma Irradiation

The predominant compounds in plant-derived calli of *E. maculata* (Figure 4) were identified using UPLC-Q-TOF/MS. The major metabolites were identified by comparing their retention times and MS data with those of authentic reference materials and previously reported data (Table 1). Moreover, the MS databases HMDB, METLIN, and ChemSpider were used to confirm the results (Figure S1). A total of 44 metabolites were identified in *E. maculata* callus, including 9 amino acid derivatives ($_{L}$-arginine, *N*-(1-deoxy-1-fructosyl)isoleucine, γ-glutamyl lysine, *N*-(1-deoxy-1-fructosyl)phenylalanine, $_{L}$-phenylalanine, γ-glutamylglutamic acid, *N*-(1-deoxy-1-fructosyl)tryptophan, $_{L}$-trytophan, and phenylalanylglycine), 2 sesquiterpenoids (pteroside B and acetylpterosin C), 16 flavonoids (epicatechin 3-glucuronide, 2′,7-dihydorxy-4′,5′-dimethoxyisoflavone, epicatechin

4′-glucuronide, kaempferol 3-rhamnosyl-6″-(4″-acetylrhamnosyl)glucoside, leucocyanidin, kaempferol 3-rhamnosyl-(6″-acetyl)galactosyl-7-glucoside, 7-glucosyl-4″glucuronoyl epigallocatechin gallate, quercetin 3,7-diglucosyl-4‴-galactoside, isoquercitrin, quercetin 3-coumaroyl-triglucoside, quercetin 3-(2‴,6‴-digalloyl)galactoside, quercetin 3-(2-galloyl)glucoside, isorhamnetin 3-rutinoyl-4′-rhamnoside, kaempferol 3-(2″-rhamnosyl-6″-acetyl)galactosyl-7-rhamnoside, kaempferol 3-feruloyl-triglucoside, and isooreientin), 7 phenolic acids (sinapic acid, gallic acid 3-*O*-gallate, quinic acid, chlorogenic acid, methyl gallate, caffeic acid ethyl ester, and 1,2,3,4,6-pentagallolyglucose), 7 lipids (dehydrophytosphingosine, lysoPC(18:3), lysoPC(18:2), lysoPC(16:0), lysoPC(18:1), phytosphingsine 1-phosphate, and PC(18:3/18:3)), and 3 chlorophylls (methyl phaephoribide B, pheophorbide A, and pheophorbide B). However, the gamma-irradiated *E. maculata* callus contained only 35 metabolites, excluding γ-glutamylglutamic acid, leucocyanidin, isoquercitrin, quercetin 3-coumaroyl-triglucoside, kaempferol 3-(2″-rhamnosyl-6″-acetyl)galactosyl-7-rhamnoside, kaempferol 3-feruloyl-triglucoside, isooreientin, phytosphingsine 1-phosphate, and methyl phaephoribide B (Table 1). After gamma irradiation, the content of the majority of phenolic derivatives (including flavonoids and phenolic compounds) decreased, but the content of γ-glutamyl lysine, *N*-(1-deoxy-1-fructosyl)phenylalanine, *N*-(1-deoxy-1-fructosyl)tryptophan, lysoPC(18:2), lysoPC(16:0), and lysoPC(18:1) significantly increased by 9.0%, 5.4%, 1.9%, 16.6%, 20.4%, and 12.9%, respectively (Figure 5).

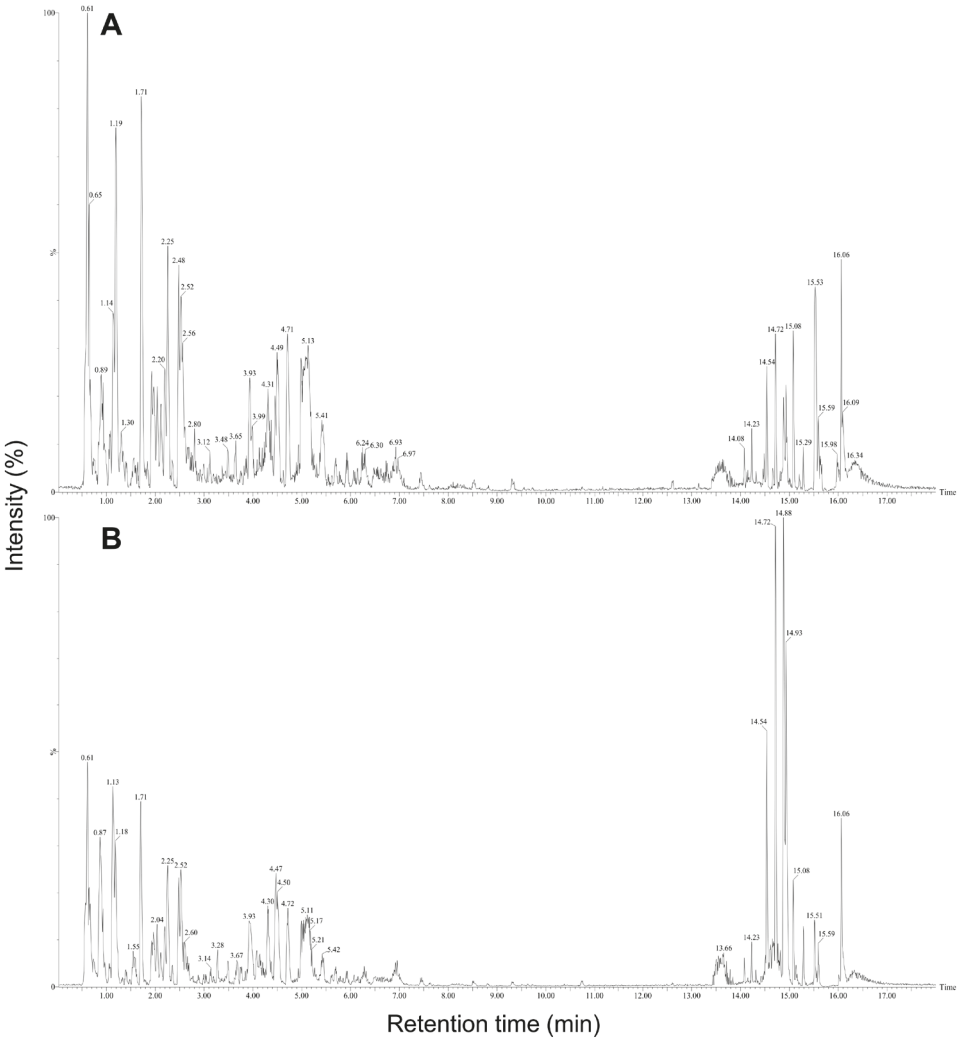


Figure 4. TIC chromatograms of non-irradiated (**A**) and irradiated (**B**) *E. maculata* calli (PC3012). Refer to the Materials and Methods section for experimental conditions.

Table 1. Metabolites identified in the non-irradiated and irradiated *Euphorbia maculata* calli using UPLC-Q-TOF/MS/MS.

Metabolite No.	RT (min)	Compounds	Exact Mass (*m*/*z*)	Fragment Ions (*m*/*z*)	Non-Irradiated	Irradiated
1	0.61	L-Arginine	175.12	70	+	+
2	0.65	Pteroside B	381.08	365, 219, 175	+	+
3	0.74	*N*-(1-deoxy-1-fructosyl)isoleucine	294.15	276, 248	+	+
4	0.87	γ-Glutamyl lysine	276.14	258, 230, 212, 147, 86	+	+
5	1.08	Epicatechin 3-glucuronide	467.08	449, 393, 153	+	+
6	1.14	*N*-(1-deoxy-1-fructosyl)phenylalanine	328.14	310, 292, 264	+	+
7	1.19	L-Phenylalanine	166.09	120, 103, 91	+	+
8	1.30	Sinapic acid	225.04	207, 153	+	+
9	1.34	Gallic acid 3-*O*-gallate	323.04	277, 259, 171, 153, 127	+	+
10	1.40	γ-Glutamyglutamic acid	277.04	259, 231, 171	+	−
11	1.59	*N*-(1-deoxy-1-fructosyl)tryptophan	367.15	349, 188, 163	+	+
12	1.71	L-Trytophan	205.10	188, 170, 159, 146, 118, 115	+	+
13	1.93	2′,7-Dihydorxy-4′,5′-dimethoxyisoflavone	315.07	297, 153	+	+
14	1.97	Epicatechin 4′-glucuronide	467.08	341, 291, 153	+	+
15	2.12	Quinic acid	193.09	149, 131, 115, 105, 103	+	+
16	2.20	Chlorogenic acid	355.10	193	+	+
17	2.25	Methyl gallate	158.04	153, 141, 123, 97	+	+
18	2.48	Caffeic acid ethyl ester	209.04	177	+	+
19	2.56	Kaempferol 3-rhamnosyl-6″-(4″-acetylrhamnosyl)glucoside	783.07	637, 619	+	+
20	2.60	Acetylpterosin C	277.04	259, 197, 171	+	+
21	2.80	1,2,3,4,6-Pentagallolyglucose	941.09	619	+	+
22	2.99	Phenylalanylglycine	223.06	177, 120	+	+
23	3.48	Leucocyanidin	307.04	275, 247	+	−
24	3.57	Kaempferol 3-rhamnosyl-(6″-acetyl)galactosyl-7-glucoside	783.09	619	+	+
25	3.65	7-Glucosyl-4″glucuronoyl epigallocatechin gallate	797.10	619	+	+
26	3.76	Quercetin 3,7-diglucosyl-4″-galactoside	789.11	771, 303	+	+
27	4.31	Isoquercitrin	465.10	303	+	−
28	4.37	Quercetin 3-coumaroyl-triglucoside	953.10	465, 303	+	−
29	4.45	Quercetin 3-(2‴,6‴-digalloyl)galactoside	769.09	617, 465, 303	+	+
30	4.71	Quercetin 3-(2-galloyl)glucoside	617.12	465, 315, 303, 297, 153	+	+
31	5.13	Isorhamnetin 3-rutinoyl-4′-rhamnoside	771.10	665, 287	+	+
32	5.31	Kaempferol 3-(2″-rhamnosyl-6″-acetyl)galactosyl-7-rhamnoside	785.09	767, 287	+	−
33	5.54	Kaempferol 3-feruloyl-triglucoside	967.12	619, 287, 177	+	−
34	5.69	Isooreientin	449.11	317, 287	+	−
35	14.23	Dehydrophytosphingosine	316.29	298, 280	+	+
36	14.48	LysoPC(18:3)	518.33	184	+	+
37	14.65	LysoPC(18:2)	520.34	184	+	+
38	14.89	LysoPC(16:0)	496.34	184	+	+
39	14.94	LysoPC(18:1)	522.35	184	+	+
40	15.08	Phytosphingsine 1-phosphate	398.23	280	+	−
41	15.20	Methyl phaephoribide B	621.27	531, 487	+	−
42	15.29	Pheophorbide A	593.28	531, 487	+	+
43	15.53	Pheophorbide B	607.29	547	+	+
44	16.06	PC(18:3/18:3)	778.54	184	+	+

+: detected; −: not detected.

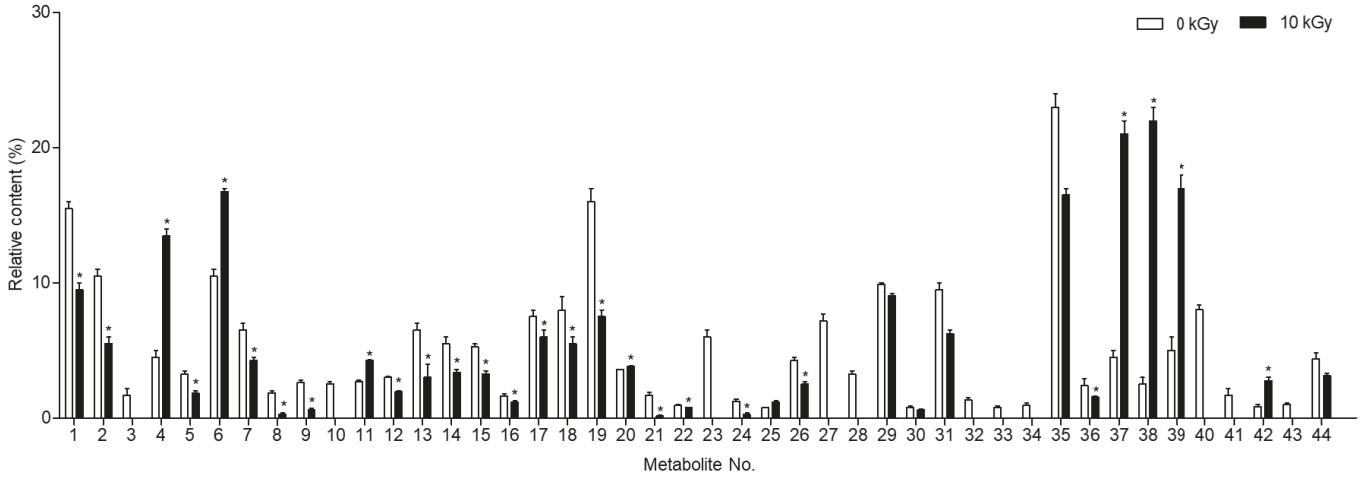


Figure 5. Changes in metabolite content (%) of non-irradiated and irradiated *Euphorbia maculata* calli (PC3012). The results are expressed as the mean ± standard deviation ($n = 3$). * $p < 0.05$ vs. the non-irradiated groups. Refer to Metabolite number in Table 1.

2.5. Statistical Analysis of Changes in E. maculata Calli after Gamma Irradiation

The PLS-DA score scatter plot and permutation test analyses (Figure 6) confirmed the significant effects of gamma irradiation on the metabolite profiles of *E. maculata* calli. The scatter plot revealed a distinct separation between non-irradiated and irradiated (10 kGy) samples, indicating substantial differences in their metabolite compositions. The permutation test supported the robustness and statistical significance of the PLS-DA model, with high R_2X (0.831) and Q2 (0.998) values and a *p*-value of 0.018 (Table S1), which confirm that the observed metabolic changes were not due to random variation but were induced by gamma irradiation.

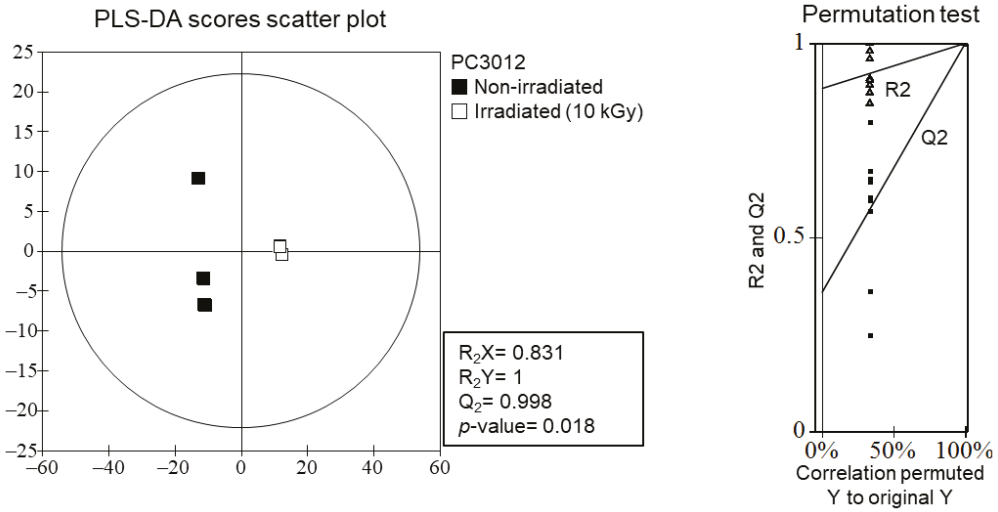


Figure 6. PLS-DA scores scatter plot and permutation test analysis of metabolite profiles of non-irradiated and irradiated *E. maculata* calli (PC3012). △: R2 values, ■: Q2 values.

3. Discussion

The primary objective of this experiment was to investigate the potential of gamma irradiation to enhance the antioxidant properties and modify the metabolite profiles of *Euphorbia maculata* callus. This study builds on previous research suggesting that gamma irradiation can induce beneficial biochemical changes in plant tissues, making it a valuable tool for improving the bioactive characteristics of plant-derived materials. The three callus extracts derived from *E. maculata*, *Punica granatum*, and *Potentilla fragarioides* exhibited

different responses to gamma irradiation. While the antioxidant activity of the *E. maculata* callus (PC0312) significantly increased, as shown by the DPPH and $ABTS^+$ assays, the *P. granatum* callus (PC3026) displayed a decrease in antioxidant activity, and the *P. fragarioides* callus (PC3062) showed only a slight improvement. These differential responses suggest that the impact of gamma irradiation on antioxidant activity is highly dependent on the specific metabolic pathways and the inherent chemical composition of each plant species. In the case of *E. maculata*, the increase in antioxidant activity may be attributed to the upregulation of specific enzymes or the enhanced synthesis of antioxidant compounds such as flavonoids and phenolic acids, which are known to be sensitive to radiation-induced stress [18]. The callus of *E. maculata* may have a robust defense mechanism that is activated under gamma irradiation, leading to an increase in the production of antioxidant metabolites. On the other hand, the decrease in antioxidant activity observed in the *P. granatum* callus could be due to the degradation of sensitive antioxidant compounds such as certain polyphenols and flavonoids, which may break down under gamma irradiation into less active or even inactive forms. This degradation might outweigh any potential increase in antioxidant activity from other newly synthesized compounds [19]. Additionally, *P. granatum* may have less efficient stress response pathways compared to *E. maculata*, leading to a net decrease in antioxidant potential following irradiation. For *P. fragarioides*, the slight improvement in antioxidant activity suggests a balance between the degradation of some antioxidants and the synthesis of others. This balance may result in a marginal increase that is not as pronounced as that observed in *E. maculata*, but still represents an enhancement over the non-irradiated state. It is possible that while certain metabolites are degraded, others are either synthesized or their activity is enhanced, leading to a slight net gain in antioxidant capacity. These findings underscore the complexity of plant responses to gamma irradiation, highlighting that the effects are not uniform across different species or even within the same metabolic pathways. The variability in response could be influenced by several factors, including the specific types of metabolites present, the structure of the callus tissues, and the efficiency of the plant's inherent stress response mechanisms. Furthermore, these results suggest that gamma irradiation may be selectively applied depending on the desired outcome, whether it is to enhance or modify the antioxidant properties of specific plant extracts. Overall, this study reveals that while gamma irradiation can be a powerful tool for enhancing the bioactive properties of certain plant calli, its effects are not universally positive and must be carefully evaluated for each specific application. Further research is warranted to explore the molecular mechanisms underlying these differential responses and to optimize irradiation conditions to achieve the desired bioactive enhancements in various plant-derived materials

The dose-dependent increase in both DPPH and $ABTS^+$ radical scavenging activities of PC3012 calli, as shown in Figure 2, further emphasizes the effectiveness of gamma irradiation in enhancing antioxidant characteristics. This suggests that gamma irradiation stimulates the synthesis or activation of antioxidant compounds in calli, with higher doses resulting in greater antioxidant activity. These findings highlight the viability of gamma irradiation as an approach to enhancing the bioactive properties of plant-derived materials, making them beneficial for pharmaceutical and nutraceutical applications [20]. Previous studies have reported that gamma irradiation affects the growth of plant-derived callus [21]. However, in this study, extraction was performed immediately after gamma irradiation without any time delay. In addition, since gamma irradiation was performed at a dose rate of 10 kGy/h, the growth time was only about 1 h at most. Therefore, it is judged that gamma irradiation did not affect the growth inhibition of callus tissue. While our current study focused on a maximum irradiation intensity of 10 kGy, we acknowledge that there is potential for further enhancement of radical scavenging activities at higher doses. This consideration has been incorporated into our discussion, and we intend to conduct future research to explore the effects of higher irradiation intensities in order to determine the optimal conditions for maximizing antioxidant activity. By exploring higher irradiation doses, we aim to gain deeper insights into the relationship between irradiation

and antioxidant capacity, which could lead to the development of more effective strategies for enhancing the bioactive properties of plant-derived materials.

The changes in metabolite content before and after gamma irradiation, as illustrated in Figure 5, indicate that gamma irradiation induces significant biochemical modifications in *E. maculata* calli. The enhanced antioxidant activity observed in irradiated calli may be attributable to these biochemical changes. Previous studies have reported that flavonoid derivatives are easily degraded by gamma irradiation, leading to the formation of compounds with strong antioxidant activity [22]. However, no new compounds were detected in the present study. Notably, we observed a substantial increase in lysophospholipid content after gamma irradiation. This finding aligns with previous research indicating that plants produce large amounts of lysophospholipids as a defense mechanism when subjected to excessive stress [23]. This suggests that gamma irradiation induces a stress response in plant calli, which results in an increase in the levels of lipid-based metabolites. To gain a deeper understanding of these changes, we performed UPLC-Q-TOF/MS analysis on the metabolite profile of *E. maculata* calli after gamma irradiation. The comprehensive analysis revealed significant changes in metabolite content that could be harnessed for practical applications. The increase in lysophospholipid content and the overall enhancement of antioxidant activity suggest that gamma irradiation can be used to improve the bioactive compounds of plant-derived calli. Previous studies have identified lysophospholipids in a wide range of tissues and cell types, where they play critical roles in various physiological functions, including vascular development, reproduction, myelination, neurological disorders, and cancer [24]. Consequently, gamma irradiation has the potential to enhance the antioxidant properties of materials, which can be beneficial for applications in the natural product and food industries [25].

The significant distinction in metabolite profiles between non-irradiated and irradiated *E. maculata* calli, as shown in Figure 6, suggests that gamma irradiation induces specific biochemical changes. While previous studies suggested that gamma irradiation can degrade flavonoid derivatives, resulting in the formation of lead compounds [22,26], our study did not detect new compounds. Instead, we observed a substantial increase in lysophospholipid content, consistent with the stress responses of plants. LC-Q-TOF/MS analysis confirmed these changes, highlighting the potential of gamma irradiation to enhance the bioactive properties of plant-derived materials. By optimizing the gamma irradiation conditions, it may be possible to create plant extracts with superior health benefits, making this a valuable technique for developing functional foods and nutraceuticals. Our study contributes to the growing body of evidence supporting the use of gamma irradiation to enhance the functional properties of natural products, thereby opening new opportunities for their application in various fields.

4. Materials and Methods

4.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^+$), methanol (MeOH), acetonitrile (MeCN), formic acid (HCOOH), and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

4.2. Gamma Irradiation of Plant-Derived Calli

Three plant-derived calli, namely *Euphorbia maculata* (PC3012), *Punica granatum* (PC3026), and *Potentilla fragarioides* (PC3062), were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) for use in the experiments (Figure S2). Gamma irradiation was conducted at room temperature following a previously established method using a cobalt-60 experimental irradiator (point source: AECL, IR-79, MDS Nordion International Co., Ltd., Ottawa, ON, Canada) at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongup, Republic of Korea [27]. The source strength at the sample position was approximately

320 kCi at a dose rate of 10 kGy/h. For dosimetry, 5 mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany) were used. The dosimeters were calibrated against an international standard set by the International Atomic Energy Agency (Vienna, Austria) to ensure accurate dose measurements. Three plant-derived callus samples (each weighing approximately 10 g) were subjected to gamma irradiation at doses of 0.05, 0.5, or 10 kGy. After irradiation, callus samples were ground and extracted overnight at room temperature using 50 mL of 80% MeOH. The extracts were filtered, and the solvent was evaporated under reduced pressure to yield crude callus extracts.

4.3. Measurement of Radical Scavenging Activity

The DPPH radical scavenging activity was assessed using the method described by Blois [28], with minor modifications. A DPPH solution (0.2 mM) was prepared in ethanol (EtOH). Each test extract was prepared at a concentration of 100 μg/mL in 70% EtOH. A 60 μL aliquot of DPPH solution was mixed with 120 μL of each test extract. The mixture was incubated in the dark for 15 min. After incubation, the decrease in absorbance was measured at 517 nm using an ELISA reader.

The $ABTS^+$ radical was generated by reacting 5 mL of $ABTS^+$ (7 mM in EtOH) with 5 mL of 2.4 mM potassium persulfate ($K_2S_2O_8$). This mixture was allowed to stand in the dark at 25 °C for 24 h before use. For the $ABTS^+$ radical scavenging assay, 100 μL of the $ABTS^+$ solution was added to a 96-well plate containing different concentrations of the callus extracts and a positive control (catechin) at 100 μM. The assay mixtures were then stirred for 30 s and incubated for 30 min at 25 °C [29]. The radical scavenging activities of DPPH and $ABTS^+$ were calculated using the following equations:

$$\text{Radical scavenging activity (\%)} = [1 - (A_2/A_1)] \times 100$$

where A_1 is the absorbance of the control (without the sample) and A_2 is the absorbance of the test sample. (+)-Catechin was used as a positive control.

4.4. High-Performance Liquid Chromatography (HPLC) Analysis

An HPLC analysis was performed using an Agilent HPLC 1200 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a photodiode array detector (PDA; 1200 Infinity series, Agilent Technologies). The system included a series of ZORBAX Eclipse Plus C_{18} columns (4.6 mm i.d. × 150 mm, particle size 3.5 μm; Agilent Technologies). The solvent system was operated in gradient mode. Initially, the mobile phase consisted of 0.1% (HCOOH) in H_2O. Over a 25 min period, the mobile phase was gradually changed to MeCN. The column temperature was maintained at 40 °C, and the flow rate was set to 1.0 mL/min. The eluting metabolites were detected using a UV detector at wavelengths of 254 and 360 nm.

4.5. Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight/Mass Spectrometry (UPLC-QTOF/MS) Analysis

For the UPLC-QTOF/MS (Xevo, Waters, Milford, MA, USA) analysis, samples (1 μL) were injected into an Acquity UPLC BEH C_{18} column (2.1 × 100 mm, particle size 1.7 μm; Waters). The column was equilibrated with H_2O containing 0.1% HCOOH (A) and eluted using a gradient with MeCN (B) at a flow rate of 0.35 mL/min. The elution program was as follows: 0–2.5 min, 85% A; 2.5–7.5 min, 80% A; 7.5–12.5 min, 70% A; 12.5–13.5 min, 100% A; 13.5–20.0 min, 0% A. The eluted metabolites were analyzed by Q-TOF/MS in the electrospray ionization (ESI) positive mode. The capillary voltage was set to 3 kV, and the sampling cone voltage was set to 30 V. The source temperature was maintained at 100 °C, with a desolvation flow rate of 800 L/h and a temperature of 400 °C. TOF/MS data were collected in the 50–1500 *m/z* range at a scan time of 0.2 s. The MS/MS spectra of the extracts were collected in the 50–1000 *m/z* range, using a collision energy ramp from 10 to 30 eV to acquire MS data including *m/z*, retention time (RT), and ion intensity. The data extraction, normalization, and alignment of the MS datasets were performed using the UNIFI software

1.8 package (Waters, Milford, MA, USA). The metabolite identification was performed using the Human Metabolome Database (HMDB; https://www.hmdb.ca, accessed on 10 March 2022), the Metabolite and Chemical Entity (METLIN) database (https://metlin.scripps.edu, accessed on 10 March 2022), ChemSpider (https://www.chemspider.com, accessed on 10 March 2022), literature references, and authentic standards.

4.6. Statistical Analysis

The MS datasets processed by the UNIFI software package were subjected to multivariate statistical analysis using SIMCA-P$^+$ version 12.0.1 (Umetrics, Umeå, Sweden). Differences among *E. maculata* callus groups were visualized using a partial least squares discriminant analysis (PLS-DA). The quality of the PLS-DA models was evaluated by the goodness of fit measures (R_2X and R_2Y) and predictive ability (Q2), and validated by cross-validation with a permutation test (n = 200). The metabolites contributing to the differences among the groups were identified based on the variable importance in the projection (VIP) value > 0.7, calculated by a PLS-DA, and with a one-way analysis of variance (ANOVA) with Duncan's test ($p < 0.05$) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The experiments were repeated at least three times to ensure consistent results. The data were expressed as the mean ± standard deviation (SD). Statistical differences between two groups were assessed using an unpaired two-tailed student *t*-test, while differences among multiple groups were analyzed using either one-way or two-way ANOVA with Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism ver. 8 (San Diego, CA, USA).

5. Conclusions

The antioxidant activity of *E. maculata* calli (PC3012) was significantly enhanced by gamma irradiation, as evidenced by the dose-dependent increase in DPPH and ABTS$^+$ radical scavenging activities. Detailed HPLC and LC-Q-TOF/MS analyses revealed no newly formed antioxidant compounds but a substantial increase in lysophospholipid content after gamma irradiation, aligned with known plant stress responses. A PLS-DA revealed clear metabolic distinctions between non-irradiated and irradiated calli, as confirmed by robust statistical validation. These findings suggest that gamma irradiation alters specific metabolites, thereby enhancing the bioactive characteristics of calli. This method has potential applications in the development of functional foods and nutraceuticals.

Supplementary Materials: The following supporting information can be downloaded from https://www.mdpi.com/article/10.3390/plants13162306/s1, Figure S1: UPLC-QTOF/MS spectra of *Euphorbia maculata* calli; Figure S2: Photographs of the three plant-derived calli; Table S1: Statistical analysis of *Euphorbia maculata* calli.

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References

1. Hrazdina, G.; Jensen, R.A. Spatial organization of enzymes in plant metabolic pathways. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 241–267. [CrossRef]
2. Real, A.; Sundell-Bergman, S.; Knowles, J.F.; Woodhead, D.S.; Zinger, I. Effects of ionising radiation exposure on plants, fish and mammals: Relevant data for environmental radiation protection. *J. Radiol. Prot.* **2004**, *24*, A123–A137. [CrossRef] [PubMed]
3. Wi, S.G.; Chung, B.Y.; Kim, J.S.; Kim, J.H.; Baek, M.H.; Lee, J.W.; Kim, Y.S. Effects of gamma irradiation on morphological changes and biological responses in plants. *Micron* **2007**, *38*, 553–564. [CrossRef]
4. Flores-Rojas, G.G.; López-Saucedo, F.; Bucio, E. Gamma-irradiation applied in the synthesis of metallic and organic nanoparticles: A short review. *Radiat. Phys. Chem.* **2020**, *169*, 107962. [CrossRef]
5. Choi, H.I.; Han, S.M.; Jo, Y.D.; Hong, M.J.; Kim, S.H.; Kim, J.B. Effects of acute and chronic gamma irradiation on the cell biology and physiology of rice plants. *Plants* **2021**, *10*, 439. [CrossRef]
6. Ahuja, S.; Kumar, M.; Kumar, P.; Gupta, V.K.; Singhal, R.K.; Yadav, A.; Singh, B. Metabolic and biochemical changes caused by gamma irradiation in plants. *J. Radioanal. Nucl. Chem.* **2014**, *300*, 199–212. [CrossRef]
7. Kim, J.H.; Lee, M.H.; Moon, Y.R.; Kim, J.S.; Wi, S.G.; Kim, T.H.; Chung, B.Y. Characterization of metabolic disturbances closely linked to the delayed senescence of Arabidopsis leaves after γ irradiation. *Environ. Exp. Bot.* **2009**, *67*, 363–371. [CrossRef]
8. Lee, K.B.; Mahlberg, P.G. Ultrastructure and development of nonarticulated laticifers in seedlings of *Euphorbia maculata* L. *J. Plant Biol.* **1999**, *42*, 57–62. [CrossRef]
9. Luyen, B.T.T.; Tai, B.H.; Thao, N.P.; Lee, S.H.; Jang, H.D.; Lee, Y.M.; Kim, Y.H. Evaluation of the anti-osteoporosis and antioxidant activities of phenolic compounds from *Euphorbia maculata*. *J. Korean Soc. Appl. Biol. Chem.* **2014**, *57*, 573–579. [CrossRef]
10. Rakotondrabe, T.F.; Fan, M.X.; Zhang, Y.L.; Guo, M.Q. Simultaneous screening and analysis of anti-inflammatory and antiproliferative compounds from *Euphorbia maculata* combining bio-affinity ultrafiltration with multiple drug targets. *J. Anal. Test.* **2022**, *6*, 98–110. [CrossRef]
11. Matsunaga, S.; Tanaka, R.; Akagi, M. Triterpenoids from *Euphorbia maculata*. *Phytochemistry* **1988**, *27*, 535–537. [CrossRef]
12. Sun, Y.; Gao, L.L.; Tang, M.Y.; Feng, B.M.; Pei, Y.H.; Yasukawa, K. Triterpenoids from *Euphorbia maculata* and their anti-inflammatory effects. *Molecules* **2018**, *23*, 2112. [CrossRef]
13. Ernst, M.; Grace, O.M.; Saslis-Lagoudakis, C.H.; Nilsson, N.; Simonsen, H.T.; Rønsted, N. Global medicinal uses of *Euphorbia* L. (Euphorbiaceae). *J. Ethnopharmacol.* **2015**, *176*, 90–101. [CrossRef]
14. Efferth, T. Biotechnology applications of plant callus cultures. *Engineering* **2019**, *5*, 50–59. [CrossRef]
15. de Oliveira, M.E.B.; Sartoratto, A.; Carlos Cardoso, J. In Vitro Calli Production Resulted in Different Profiles of Plant-Derived Medicinal Compounds in Phyllanthus amarus. *Molecules* **2020**, *25*, 5895. [CrossRef]
16. Asyakina, L.; Ivanova, S.; Prosekov, A.; Dyshlyuk, L.; Chupakhin, E.; Ulrikh, E.; Babich, O.; Sukhikh, S. Determination of the qualitative composition of biologically active substances of extracts of in vitro callus, cell suspension, and root cultures of the medicinal plant *Rhaponticum carthamoides*. *Appl. Sci.* **2021**, *11*, 2555. [CrossRef]
17. Kwon, S.U.; Cha, J.Y.; Lee, H.Y.; Xin, M.; Ji, S.J.; Kim, D.K.; Park, D.S.; Pyo, M.K.; Lee, Y.M. Chloroform fraction of *Euphorbia maculata* has antiplatelet activity via suppressing thromboxane B_2 formation. *Mol. Med. Rep.* **2015**, *11*, 4255–4261. [CrossRef] [PubMed]
18. Song, H.P.; Kim, D.H.; Jo, C.; Lee, C.H.; Kim, K.S.; Byun, M.W. Effect of gamma irradiation on the microbiological quality and antioxidant activity of fresh vegetable juice. *Food Microbiol.* **2006**, *23*, 372–378. [CrossRef]
19. Jan, S.; Parween, T.; Siddiqi, T.O.; Mahmooduzzafar. Effect of gamma radiation on morphological, biochemical, and physiological aspects of plants and plant products. *Environ. Rev.* **2012**, *20*, 17–39. [CrossRef]
20. Lee, S.S.; Lee, E.M.; An, B.C.; Kim, T.H.; Lee, K.S.; Cho, J.Y.; Yoo, S.H.; Bae, J.S.; Chung, B.Y. Effects of irradiation on decolourisation and biological activity in *Schizandra chinensis* extracts. *Food Chem.* **2011**, *125*, 214–220. [CrossRef]
21. Azeez, H.; Ibrahim, K.; Pop, R.; Pamfil, D.; Hârţa, M.; Bobiș, O. Changes induced by gamma ray irradiation on biomass production and secondary metabolites accumulation in *Hypericum triquetrifolium* Turra callus cultures. *Ind. Crop. Prod.* **2017**, *108*, 183–189. [CrossRef]
22. Jeong, G.H.; Lee, H.; Lee, H.K.; Choi, H.J.; Chung, B.Y.; Bai, H.W. Inhibitory effect of γ-ray-modified hydroxymethylated baicalins on NO production. *Bioorg. Med. Chem. Lett.* **2023**, *96*, 129491. [CrossRef] [PubMed]
23. Colles, S.M.; Chisolm, G.M. Lysophosphatidylcholine-induced cellular injury in cultured fibroblasts involves oxidative events. *J. Lipid Res.* **2000**, *41*, 1188–1198. [CrossRef] [PubMed]
24. Rivera, R.; Chun, J. Biological effects of lysophospholipids. *Rev. Physiol. Biochem. Pharmacol.* **2008**, *160*, 25–46. [CrossRef] [PubMed]
25. De Maria, L.; Vind, J.; Oxenbøll, K.M.; Svendsen, A.; Patkar, S. Phospholipases and their industrial applications. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 290–300. [CrossRef] [PubMed]
26. Jeong, G.H.; Kim, T.H. Hydroxymethylation of rutin induced by radiolysis as novel α-glucosidase inhibitors. *Chem. Pharm. Bull.* **2017**, *65*, 678–682. [CrossRef]
27. Jeong, G.H.; Cho, J.H.; Jo, C.; Lee, S.; Lee, S.S.; Bai, H.W.; Chung, B.Y.; Kim, T.H. Gamma irradiation-assisted degradation of rosmarinic acid and evaluation of structures and anti-adipogenic properties. *Food Chem.* **2018**, *258*, 181–188. [CrossRef] [PubMed]

28. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199–1200. [CrossRef]
29. Nenadis, N.; Wang, L.F.; Tsimidou, M.; Zhang, H.Y. Estimation of scavenging activity of phenolic compounds using the $ABTS^{\bullet+}$ assay. *J. Agric. Food Chem.* **2004**, *52*, 4669–4674. [CrossRef]

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Aristolochia clematitis L. Ethanolic Extracts: In Vitro Evaluation of Antioxidant Activity and Cytotoxicity on Caco-2 Cell Line

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Abstract: *Aristolochia* sp. plants are used in traditional medicine because of their immunostimulatory and anticarcinogenic properties, despite their content of aristolochic acids (AAs), carcinogenic and nephrotoxic agents. Therefore, ethanolic extracts of *Aristolochia clematitis* leaves, a specie growing in Western Romania, were obtained to study antioxidant and cytotoxic effects. The antioxidant capacity of the extract was evaluated by five in vitro chemical-based assays, proving that ABTS assay was a better method for this type of evaluation showing an IC_{50} of 160.89 ± 0.21 μg/mL. Furthermore, the cytotoxic effects of the extract were established by an IC_{50} of 216 μg/mL for 24 h by MTT assay, followed by a cell-based assay on Caco-2 cells by the ABTS method. The antioxidant effects of the *A. clematitis* extract demonstrate potential therapeutic applications in complementary medicine.

Keywords: *Aristolochia* sp. plants; antioxidant capacity; ethanolic leaves extract; colon cancer cell line

1. Introduction

Medicinal plants were the first types of treatment for health conditions since ancient times for a wide spectrum of diseases and medical conditions. Recognizing the medicinal significance of indigenous plants, the World Health Organization (WHO), in its 1997 guideline, stated that "effective locally available plants can be used as substitutes for drugs" [1]. Nowadays, almost 50% of new drugs have been patterned after natural phytochemical compounds [1]. Interest in the development of innovative natural remedies based on medicinal plants to protect human health has increased, especially in developing countries, by over 80% during the last decades [2,3].

In Europe, Asia, and South America, *Aristolochia* spp. has been utilized as herbal medicine for more than 2000 years. *Aristolochia* sp. plants are useful in treating a wide range of conditions, as they have anti-inflammatory, antioxidant, antimicrobial and immunostimulatory properties [4,5], as well as medicinal effects that have been thoroughly demonstrated to be due to a wide array of bioactive compounds [6]. On the other hand, these plants were forbidden to be used due to their content in aristolochic acids (AAs), nephrotoxic, carcinogenic, and mutagenic compounds [7,8]. The short-term exposure (up to one year) to AAs lead to aristolochic acid nephropathy (AAN) [9–13], and a longer term exposure (up to several decades) can lead to Balkan endemic nephropathy (BEN) [14,15]. Both of these kidney diseases are associated with urothelial carcinoma [16], and with similar clinical expression and pathological lesions [16].

Even if these plants were forbidden by the US Food and Drug Administration and regulatory authorities of some countries [17], it is still used in the form of drugs or phytomedical preparations [7,18], especially in Asia [1], due to the beneficial effects of their short-term

consumption [2,19–21]. The beneficial effects of bioactive molecules are attributed to secondary compounds such as alkaloids, terpenoids and polyphenolic compounds [7,8,22], these compounds being powerful antioxidants that fight free radicals, which result from respiration and metabolic processes [20]. Free radicals resulting from AAs' effects are reactive and hence, oxidative molecules and their overproduction can lead to oxidative stress, which is linked to inflammation, kidney disease, and is associated with cancers [7,8,23].

An understanding of the biological properties of *Aristolochia clematitis*, the most frequent species found in Europe [7,18,23], can facilitate the identification of compounds with therapeutic potential. The *Aristolochia* species were already screened for their chemical composition; therefore, secondary metabolites, classified according to chemical structure, including aristolochic acids and esters, aristolactams, aporphines, protoberberines, isoquinolines, benzylisoquinolines, amides, flavonoids, lignans, biphenyl ethers, coumarins, tetralones, terpenoids, benzenoids, and steroids have been thoroughly characterized [5,24–26]. Other classes of compounds were identified by gas-chromatography analyses from *A. tagala* leaves extracted in different types of solvents, hydroalcoholic and methanolic, showing the most diverse biologically potent compounds (e.g., alkaloids, flavonoids) [27]. Also, *A. clematitis* from Romania, prepared as tincture and infusion from roots, steams, leaves was analyzed by two types of gas-chromatography revealing various classes of compounds, from fatty acids to terpenic compounds, coumarins and alkaloids, and aristolochic acids derivatives, all with therapeutic activities [28]. Another study on *A. clematitis*, but from Algeria, showed that organic solvents and water extracts from roots possess similar composition (e.g., terpenoids, alkaloids, fatty acids) [29]. These compounds could aid in developing new drugs with antioxidant capabilities, potentially treating various conditions linked to oxidative stress [30], while encouraging the sustainable exploitation of plant resources [31]. The extraction process to obtain bioactive substances are preferentially carried out using ethanol as an extraction solvent, given its high affinity for both lipophilic and hydrophilic bioactive molecules. Also, ethanol is the most commonly used solvent because of its economical and reusability value as well as low toxicity [32]. Limited studies on ethanolic extract from *A. clematitis* aerial parts demonstrated through several chemical-based antioxidant assays, that the extract had good antioxidant activity with polyphenolic and flavonoids content methods [33]. Chemical-based assays are preferred to be used in an initial screening process of the natural products because they are low cost, simple and rapid procedures, with cheap reagents, and require simple instrumentation (e.g., spectrophotometer) [34,35].

The major pathway for herbal products containing AAs to the kidneys, where the effects are irreversible by long-term exposure, is through the digestive system, after absorption and passage through the intestinal cells; these cells are vulnerable to oxidative damage due to the exposure to luminal oxidants and toxins from ingested foods [36,37]. Medicinal plants are used in the treatment of colon cancers [38], their effects being investigated on various human cell lines derived from the intestinal mucosa. Caco-2 is such a cell line derived from a human colorectal adenocarcinoma, and represents a reliable proxy model of intestinal absorption of AAI [37,38].

Our study aimed to investigate in more detail the in vitro antioxidant properties and in vitro cytotoxic activity of the *Aristolochia clematitis* extract. As such, we used dried leaves as biological material and had them extracted at room temperature by maceration with ethanol, which has a similar formulation with the one used in ethnomedicine. The extract was used for the evaluation of antioxidant activities with five different chemical assays. Four out of the five assays were chosen to compare our extract with the literature data: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), total antioxidant capacity (TAC), total flavonoids content (TFC) and total polyphenolic content (TPC). The ABTS assay was chosen as a different method; to our knowledge this method was used for the first time in the literature to test *Aristolochia* sp. extracts or compounds for antioxidant activity. Also, the ABTS method can be used both as a chemical based-assay and cell-based assay. Before testing the extract's antioxidant activity on the Caco-2 cell line, the extract's toxicity was

evaluated to establish an inhibitory effect. After the concentration of the extract, which inhibited 50% of the cells, was established, an antioxidant effect by the ABTS assay was evaluated. The sequence of the experimental design is depicted in Figure 1.

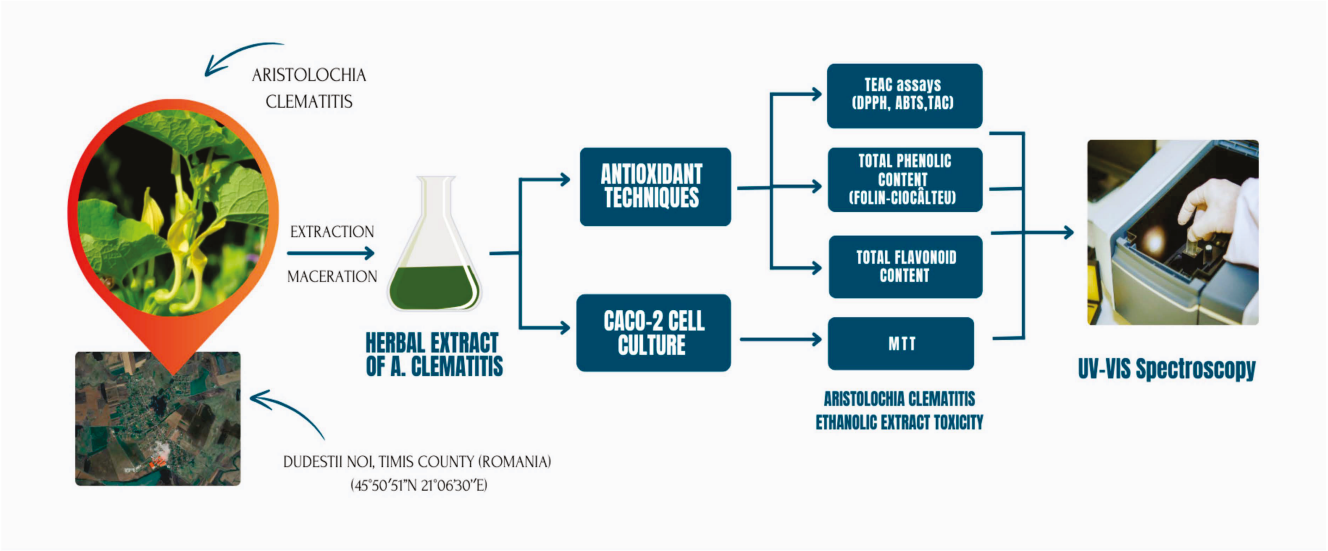


Figure 1. A synoptic view of the experiments performed.

2. Results

2.1. The Antioxidant Activities of A. clematitis Leaf Ethanolic Extract

To assess the antioxidant potential of *A. clematitis* leaf extracts, five distinct assays were employed, and three of them used Trolox as standard; the findings delineating the antioxidant capacity are detailed in Table 1. Antioxidants are able to reduce the color intensity of DPPH, ABTS, and phosphomolybdate solutions due to their ability to donate protons; these effects have been quantified using as standard, the Trolox equivalent antioxidant capacity. The IC_{50} values for DPPH and ABTS were calculated by graphing concentrations of the extract against the proportional antioxidant inhibition percent [39,40]. Another Trolox method used was the total antioxidant capacity (TAC) using the phosphomolybdate reagent [41,42]. The other two methods were based on propyl gallate and quercetin calibration curves and the spectrophotometric measurement of the total polyphenolic content and the total flavonoid content. Results are obtained from three independent experiments, with each sample plated in quadruplicate; these are presented in Table 1.

Table 1. The antioxidant activities of *A. clematitis* ethanolic extract tested with five different assays.

Samples	TEAC IC_{50} (µg/mL)			TPC (mg GAE/g DW)	TFC (mg Q/g DW)
	DPPH	ABTS	TAC		
AcE	392.62 ± 0.06	160.89 ± 0.21	4816.37 ± 0.03	3.55 ± 0.006	60.83 ± 0.01
Trolox	393.95 ± 0.19	157.90 ± 0.18	4271.28 ± 0.002	-	-

Comparing the DPPH and ABTS results, the first method gave values almost three times higher than the ABTS ones. The extract sample values were similar to those of Trolox for each of the methods. The TAC method gave values almost 10 times higher than DPPH and 25 times higher than ABTS. An IC_{50} value more than 150 µg/mL represents weak antioxidant activity [43]. This indicates that the ABTS method could be a better method to demonstrate the antioxidant activity of the ethanolic extract. The total phenolic content has a low value, showing a low content in polyphenols, but the total flavonoid content has a high activity suggesting a high content in flavonoids. Therefore, a correlation between TPC and TFC assays shows that the method of extraction with ethanol at room temperature is a proper method for flavonoids extraction.

2.2. Evaluation of Cytotoxicity of Ethanolic Extract on Caco-2 Cells

The effects of Trolox and extracts on Caco-2 cells were evaluated by the MTT assay. Five dilutions of *A. clematitis* ethanolic extracts of 100, 200, 300, 500, and 600 μg/mL were used to obtain an IC_{50} for both 24 and 48 h. The same procedure was used for the calculation of the IC_{50} of Trolox for the three different concentrations ranging from 0.5 to 0.125 mg/mL for 24 h. The results obtained by the MTT assay for 24 h and 48 h were 216 μg/mL and 224 μg/mL, respectively, and 207 μg/mL for Trolox. The cells were photographed for morphological changes at 100x magnification (Figure 2).

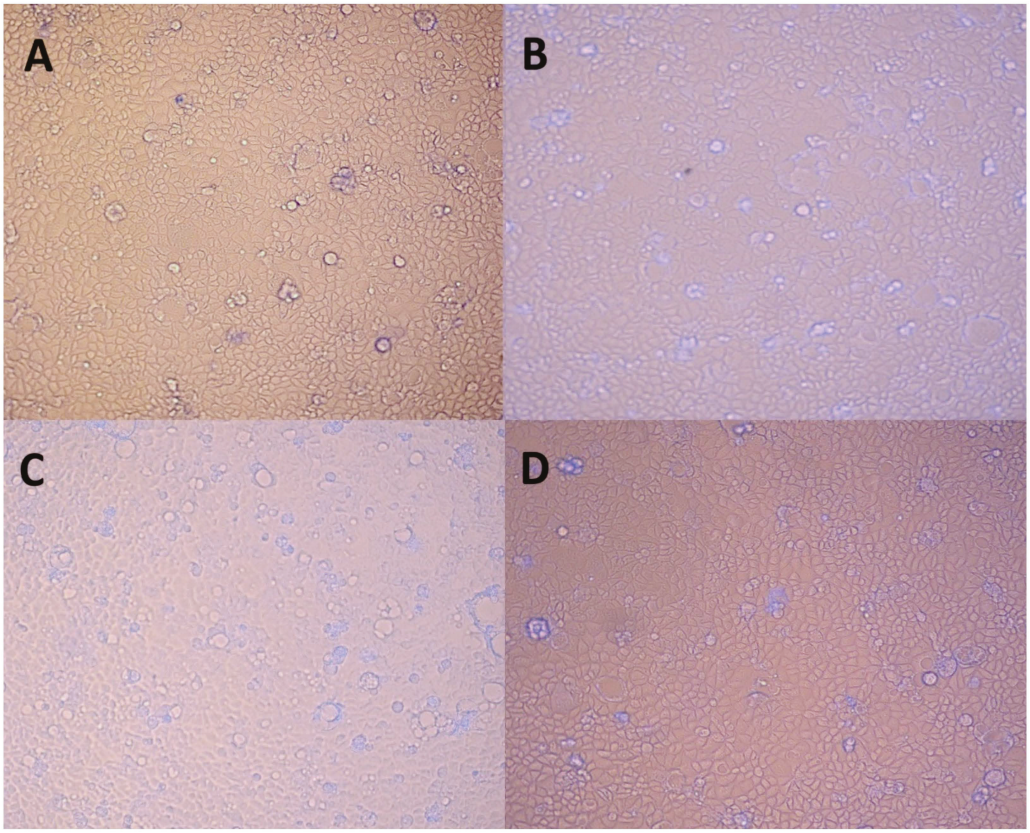


Figure 2. Morphological assessment of Caco-2 cells (control vs. treatment after 24 h of incubation) exposed to (**A**). AcE (150 μM), (**B**). Trolox (2 mM), (**C**). ethanol, and (**D**). control cells.

The Caco-2 cells did not show any morphological differences upon exposure to the *Aristolochia* extract or Trolox, with the effect being similar (Figure 2C,D). These results suggest that the short-term exposure of Caco-2 cells to the extract does not affect their morphology, at least at the tested concentration.

2.3. Evaluation of Antioxidant Capacity on Caco-2 Cells by ABTS Assay

Antioxidant activity with the ABTS method was performed after establishing an IC_{50} value and the chosen concentration for the ABTS assay was 150 μM for ethanolic extract, and 2 mM for Trolox. The antioxidant activity in Caco-2 cells was assessed by the ABTS assay [40]. Antioxidant activity was expressed as mg Trolox Equivalents (TE)/10^6 cells. Results obtained by the ABTS method were compared (Figure 3).

Ethanolic extract-treated cells had an antioxidant activity closer to Trolox, and control cells had closer results to ethanol treated ones. Samples were compared by single factor ANOVA statistical analysis and only cells treated with the extract exhibited statistically significant antioxidant activity compared to the control group ($p < 0.05$).

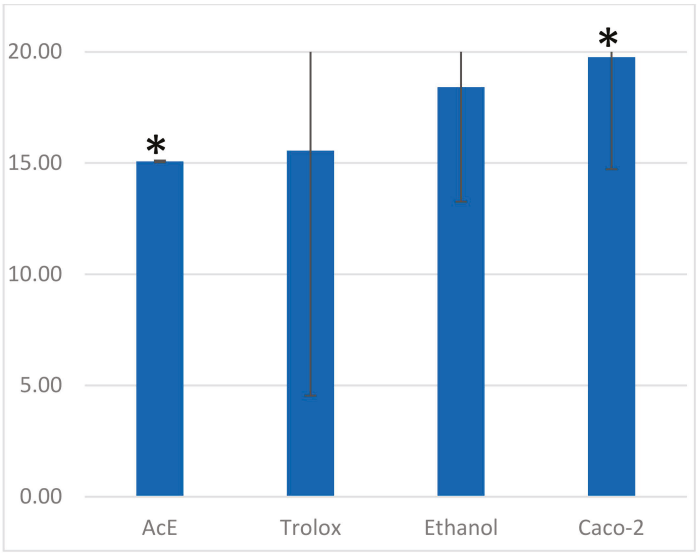


Figure 3. Graphical representation of the ABTS results obtained on Caco-2 cells treated with *A. clematitis* ethanolic extract (AcE, 150 µM); Trolox (2 mM); ethanol as the solvent control; and Caco-2 absolute control cells. *—samples statistically different ($p < 0.05$).

3. Discussion

Human health is affected by oxidative stress, which appears as an imbalance between oxidants and antioxidants in favor of oxidants [44]. Regarding the fact that in the present study a natural product was evaluated, chemical and cell-based assays were used. Through these methods, different aspects of the compounds reactivity toward ROS was evaluated [45]. This study contributes to the literature with new data on *A. clematitis* ethanolic extracts and their analysis on some new antioxidant assays. In the scientific literature, the antioxidant assays employed were total phenolic content, flavonoid content, reducing power, ferrous metal ions chelating, superoxide anion, hydroxyl radical, hydrogen peroxide, and nitric oxide [33,46]. Based on Lerma et al. (2022), other species of *Aristolochia* extracts were evaluated for their antioxidant and anticancer activity [23].

A. clematitis ethanolic extracts demonstrated promising DPPH radical scavenging activity with an IC_{50} value of 391.62 ± 0.06 µg/mL, compared to the IC_{50} values for the standard Trolox of 393.95 ± 0.19 µg/mL. The free radicals scavenging activities for *A. clematitis* organic ethanolic extract gave a percentage inhibition of 69.58 ± 0.07, almost three times higher than the one obtained by Papuc et al. (2010) [33], which exhibited a DPPH inhibition of 24.77 ± 3.28 [33]. Other studies showed antioxidant activity with the DPPH method on other *Aristolochia* species, for instance *A. baetica* and *A. paucinervis* methanolic extracts, which present IC_{50} values of 150 ± 8 µg/mL and 160 ± 10 µg/mL, respectively, when compared to ascorbic acid [47], and *A. longa* organic extract with an IC_{50} value of 125.40 ± 2.40 µg/mL [48]. *A. clematitis*'s capacity to scavenge DPPH radicals has demonstrated high value, indicating that the extract has low antioxidant activity because it requires a larger amount to achieve the desired level of radical scavenging [49]. Another specie of *Aristolochia*, *A. tagala*, presented antioxidant effects when leaves were extracted in a hydroalcoholic solvent system (30.80 µg/mL) [27]. A study on *A. clematitis* roots methanolic extracts from Algeria showed an IC_{50} of 142 µg/mL [29], and its antioxidant capacity was attributed to the active compounds and the synergy among them.

To the best of our knowledge, no previous data were available for the *A. clematitis* ABTS assay. An IC_{50} for the herbal extract was estimated at 160.89 ± 0.21 µg/mL, and a value of 157.90 ± 0.18 µg/mL was obtained for Trolox, respectively; in other experiments performed on *A. bracteata* ethanolic extracts, an IC_{50} value of 19.42 µg/mL for ABTS scavenging activity was obtained [50].

The total polyphenolic content of *A. clematitis* was quantified after an adapted Folin–Ciocâlteu method [51]. The ethanolic extract of *A. clematitis* has been shown to have a total polyphenolic content of 3.55 ± 0.006 mg GAE/g. One study presents the TPC of an ethanolic extract obtained from rhizomes and the results were 91.3 ± 4.75 mg GAE/g of extract [52]. Another study on phenolic content performed on *A. clematitis* aerial parts extracted in 60% ethanol revealed a 4.6 ± 1.88 mg caffeic acid equivalent/100 mL extract [46]. Additional research showed that the total polyphenolic content of two other species of *Aristolochia* was estimated at 360 ± 20 mg GAE/g and 280 ± 27 mg GAE/g [47]. In another study, the phenolic content determined on *A. talaga* showed a value of 52.96 ± 0.04 mg GAE/g [53], and on a hydroalcoholic and methanolic type of extract, values around 50 mg GAE/g extract were obtained [27]. The low phenol content suggests that other molecules with a different polarity, likely generated by hydrolysis, and other cleavage events may be responsible for the high antioxidant effect that has been experimentally obtained.

The total flavonoid content of *A. clematitis* was estimated at 60.83 ± 0.01 mg QE/g. Also, in another study on *A. clematitis* performed on rhizomes ethanolic extract obtained at 35 °C, the results were five times higher than ours (295.8 ± 12.6 mg QE/g plant) [52]. Also, Crivineanu et al. (2009) used *A. clematitis* aerial parts 60% ethanolic extract and obtained a value of 13.35 ± 3.17 μg QE/mL [46]. The experiment described by Saeed et al. was followed in order to determine the total flavonoid content of *A. clematitis* [54]. Our results were consistent with those described in previous research, where the flavonoid content in *A. tagala* methanolic extract was estimated at 51.65 ± 0.11 mg QE/g [53], and around 50 mg QE/g in hydroalcoholic and methanolic leaf extracts' TFC [27].

The total antioxidant capacity of plant extracts has been routinely evaluated using the phosphomolybdate method [41]. The TAC of the extracts was evaluated at 4.82 ± 0.03 mg/mL. A higher absorbance value indicates a higher level of antioxidant activity [55]. This method of total antioxidant capacity was performed for the first time for this *Aristolochia* sp. In the current literature, the phosphomolybdenum method is used for aristolochic acids isolated from *A. bracteolata* leafs and stems methanolic extract [42].

The toxicity of *Aristolochia* extracts on Caco-2 cells was investigated. The obtained IC_{50} for both exposure timeframes, 24 h and 48 h, suggest that the extracts are only weakly active on Caco-2 cells at these chosen concentrations. Our results showed cytotoxic effects almost similar for both timeframes and concentrations, 216 μg/mL at 24 h and 224 μg/mL at 48 h, respectively, and closer to Trolox 207 μg/mL.

In different studies, aqueous and organic extracts from *Aristolochiaceae* family plants were evaluated for cytotoxic activity, by MTT or sulforhodamine B (SRB) colorimetric assays, on human cancer cell lines. The difference between cytotoxic activities of *Aristolochia* sp. extracts can be explained by several parameters: the specific particularities of the *Aristolochia* plants; the part of the plant used in the study; the different composition of extracts due to the use of different aqueous or organic solvents; the type of extraction; and the particular features of the cancer cell line. Table 2 encompasses the results of investigations from the literature into the effects of organic extracts based on ethanolic solvents from various species of the genus *Aristolochia* on different cancer cell lines.

The ethanolic extract of the *A. ringens* root was significantly active for all cancer cell lines with IC_{50} values between 3 and 24 μg/mL [19]. *A. galeata* ethanolic extract obtained from rhizomes by maceration for 10 days at room temperature and a hydroalcoholic fraction had the lowest cytotoxicity on the HeLa cell line by the MTT assay after 72 h. Only the organic dichloromethane fraction presented cytotoxic effects [56]. In other experiments, *Aristolochia bracteolata* Lam. leaves were extracted not only with ethanol, but also in combination with other solvents, like methanol and ethyl acetate, and their cytotoxicity was tested by the MTT assay on the Vero cell line and A549 adenocarcinoma cell line. The extract had a cytotoxic effect on cancer cells at a low concentration, while a higher concentration induced cell growth [32]. On the HCT-15 cell line, and its multidrug-resistant subline, HCT-15/CLO2, the extract exhibited potent cytotoxic activities with growth inhibition (GI_{50}) values in the micromolar range (>10 μM) [57]. According to current research,

the most potent cytotoxic effects of *Aristolochia* sp. come from extracts obtained in different organic solvent systems (<100 μg/mL) [58–63].

Table 2. The IC_{50} values of organic extracts including alcoholic extracts of the *Aristolochiaceae* family.

Cell Line	Species	Part of the Plant	Type of Extract	Type of Solvent	IC_{50} (μg/mL)	Ref
A549 (lung) HCT-116 (colon) PC3 (prostate) A431 (skin) HeLa (cervix) THP-1 (leukemia)	*A. ringens* Vahl.	root	macerated	EtOH/HydroEtOH/EtOH + HydroEtOH/DCM-MeOH	20/ > 100/ > 100/26 22/ > 100/ > 100/19.5 3/ > 100/ > 100/12 -/ > 100/ > 100/28 -/ > 100/ > 100/30 24/ > 100/ > 100/22	[19]
HeLa	*A. galeata* Mart.	rhizomes	macerated	EtOH/hexane/DCM/ethyl acetate/hydroEtOH	369/726/90/1620/1340	[56]

EtOH—ethanol; DCM—dichloromethane; and MeOH—methanol.

Furthermore, Trolox showed a higher antioxidant effect on Caco-2 cells compared to the extract (Figure 3). This type of assay was implemented for the first time in the present study. Trolox was used as a control compound because of its use in the calibration curve calculations and because of its use in antioxidant capacity evaluations of *A. clematitis* extracts. This type of cell line was used because of its good reproducibility and predictability of antioxidant activities in vivo [64]. *Aristolochia* sp. extracts and Trolox have in vitro antioxidant activities, and these can be inhibited by low doses of aristolochic acids present in leaves. AAs have cytotoxic effects and their presence in extracts induces ROS production by activating the caspase pathway [65]. *A. debilis* stems methanolic extracts demonstrated dose–time inhibiting effects, on the HT-29 human colon cancer cell line, due to ROS overproduction [66].

This study brings into light new data which are useful especially for alternative medicine approaches, as well as for toxicologists and physicians who diagnose and treat patients with AA-induced kidney injuries. *Aristolochia* sp. extracts can also be used in nanomedicine by synthesizing new types of silver nanoparticles [67,68]. Information about *Aristolochia clematitis* products is available on the website of the Natural Medicines Comprehensive Database [69]. Therefore, data about the safety of medicinal products are presented in the current system of monitoring but the adverse effects are incompletely described. The risks associated with the use of *Aristolochia* sp.-based herbal medicinal products can be reduced by their short-term use as complementary treatment in inflammatory diseases and cancer associated with gastrointestinal tract [3]. Further interdisciplinary collaboration between research scientists and physicians could lead to improved phytotherapeutically formulas by refining extraction protocols and removing the phytotoxins, and guarantee secure and effective integration of these medicinal plants into contemporary therapeutics.

4. Materials and Methods

4.1. Chemicals and Reagents

Analytical grade ethanol purchased from Sigma-Aldrich (Merck Group, Darmstadt, Germany) was used for extractions and dilutions. For the antioxidant activity assays, the following reagents were used: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 1,1-diphenyl-2-picrylhydrazyl radical (DPPH); 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2S_2O_8$); ammonium molybdate tetrahydrate, sodium phosphate dibasic, sulphuric acid; and Folin–Ciocâlteu reagent, sodium carbonate, propyl gallate; sodium nitrite ($NaNO_2$), aluminum chloride hexahydrate ($AlCl_3$*$6H_2O$), sodium hydroxide (NaOH), all purchased from Sigma-Aldrich (Merck Group, Darmstadt, Germany).

4.2. Raw Material Plant Collection and Extraction

Aristolochia clematitis L. plants were harvested from Dudeştii Noi, Timiş County (Romania) (45°50′51″ N 21°06′30″ E) in September 2023. A voucher specimen (CBG-AcL-01) has been identified by a botanist and the plant was stored in the Herbarium of the Faculty of Chemistry, Biology, Geography from Timișoara, Romania. The collected specimens were air-dried in the dark at room temperature (RT) and only leaves were ground and used in the extraction process. One gram of leaves was mixed with 15 mL of ethanol and left for maceration under a continuous homogenization at RT for 24 h. *Aristolochia clematitis* ethanolic extract samples (AcE) were centrifuged at 3000 rpm for 5 min and 14 ± 0.3 mL of supernatant was collected, filtered through a 0.45 µm polyethersulfone (PES) filter (Merck Group, Darmstadt, Germany), and kept at 4–8 °C for future analyses.

4.3. Antioxidant Activity Evaluation Techniques

4.3.1. The Trolox Equivalent Antioxidant Capacity (TEAC) Assay

For TEAC assays, DPPH, ABTS, and TAC, respectively, were used and 2 mM Trolox was used as a positive standard for calculation. To obtain a calibration curve, dilutions ranging from 0.5 mg/mL to 3 µg/mL were used. As a negative control, we used the specific extraction solvent, ethanol. Also, dilutions of extracts were performed to calculate the inhibition percentage and further, the obtained calibration curve was used to calculate the IC_{50}. Therefore, the IC_{50} (µg/mL) value, defined as the concentration of extract required to scavenge the formation of 50% of free radicals, was calculated using Excel software Version 130.0.2849.52 (Microsoft Corporation, Redmond, WA, USA) and expressed as mean ± standard deviation (SD) of three independent experiments. Samples and standards were plated in quadruplicates on 96 round-bottom transparent well plates and their absorbances were registered on a spectrophotometer Tecan i-control, 1.10.4.0 infinite 200Pro. The particularities for each type of TEAC assays are described below.

DPPH Radical Scavenging Assay

DPPH radical scavenging assay was conducted using the stable radical, DPPH [39]. A 2.54 mM DPPH methanolic stock solution was used for a calibration curve including concentrations ranging from 0.1 mg/mL to 3.1 µg/mL. Plant extracts diluted 1:10 in the specific solvents were mixed in a ratio of 1:8 (*v*/*v*) with 0.25 mM DPPH methanolic solution and incubated in the dark at room temperature. After a 30 min reaction period, samples were registered at 517 nm. The following equation was used to compute the DPPH scavenging activity:

$$\%\text{DPPH scavenging activity} = (A_{control} - A_{AcE}) \times 100/A_{control},$$

where $A_{control}$ and A_{AcE} are the absorbances of the control, ethanol, and the extract.

ABTS Radical Scavenging Assay

ABTS radical scavenging activity was conducted using an adapted method [63]. ABTS (1 M) and $K_2S_2O_8$ (2.45 mM) were combined 1:1 (*v*/*v*) in an amber-colored bottle for the preparation of the ABTS cation ($ABTS^+$) and kept in the dark at RT for 14 h. The obtained $ABTS^+$ solution was diluted with the specific solvents for each type of extract (1:20) to an absorbance of 0.700 ± 0.035 at 734 nm. Subsequently, 300 µL of $ABTS^+$ solution was mixed with 100 µL of each extract and left at RT in the dark for 30 min; absorbances were measured at 734 nm. The following equation was used:

$$\%\text{ABTS+• inhibition} = (A_{control} - A_{AcE}) \times 100/A_{control},$$

where $A_{control}$ measures the absorbance ABTS+• solution mixture without adding the sample, and A_{AcE} measures the absorbance of the sample extract and ABTS+• solution mixture.

Total Antioxidant Activity (TAC) Assay

The total antioxidant capacity (TAC) of samples was determined by the method described by Prieto et al., 1999 [41]. The assay is based on the reduction of Mo(VI) to Mo(V) by samples and formation of a green-colored phosphate/Mo(V) complex. A ratio of 1:5 (*v*/*v*) sample to reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate dibasic and 4 mM ammonium molybdate tetrahydrate was combined in Eppendorf test tubes. The test tubes were incubated at 95 °C for 90 min to complete the reaction, and then cooled at RT to measure absorbances at 695 nm.

4.3.2. Total Phenolic Content Assay (TPC)

The total phenolic content (TPC) of the samples was performed with an adapted Folin–Ciocâlteu method, as previously described with minor modifications [70,71]. Briefly, 1 mL of each extract was mixed in a 1:5 ratio sample with Folin–Ciocâlteu reagent (diluted 1:10 in distilled water to obtain a 0.25 N concentration) and left for five minutes in the dark at room temperature; after this, an equal volume of 7.5% sodium carbonate solution with Folin–Ciocâlteu reagent was added, mixed, and left in the dark at RT for 1 h. The absorbance of the samples was measured at 725 nm. TPC was expressed in gallic acid equivalents (mg GAE/g extract) calculated after a propyl gallate calibration curve with concentrations between 0.15 mg/mL and 1.2 μg/mL.

4.3.3. Total Flavonoid Content Assay (TFC)

The total flavonoid content (TFC) of different extracts was determined by a colorimetric method using aluminum chloride ($AlCl_3$) [72]. One mL of each extract was mixed sequentially in 30% methanol with aqueous solutions of 0.3 M $AlCl_3$*$6H_2O$, 0.5 M $NaNO_2$, and 1 M NaOH. The mixtures were kept in the dark at RT for 10 min and after that, the absorbance was measured at 510 nm. The concentration of flavonoids was calculated using a quercetin standard curve (2.5 mg/mL to 78 μg/mL) and expressed as milligrams of quercetin equivalent (QE) per gram of dried leaves (mg QE/g dried leaves).

4.4. In Vitro Assays

4.4.1. Caco-2 Cell Cultures

Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and expanded in DMEM cell culture media. When the cell culture reached 80% confluence, cells were dispersed with 0.025 M trypsin-EDTA and reseeded in a new flask. The passage number of the cells used in the experiments was between 25 and 39. The culture medium was replaced every 48 h. Cells were cultivated with the specific culture media which included Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DMEM-F12), supplemented with 20% Fetal Calf Serum and 1% mixture of Penicillin/Streptomycin. All cultures were plated and incubated at 37 °C with 5% CO_2. Caco-2 cells were left in culture plates to proliferate until a confluence of 90% was reached and were subsequently split into culture plates for further assays.

4.4.2. MTT Assay

A. clematitis ethanolic extract (AcE) and Trolox effects on Caco-2 cells were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction (MTT) assay (Sigma-Aldrich, Merck Group, Darmstadt, Germany). Briefly, Caco-2 cells, passage 5, and 7 were seeded in 96-well plates (7000 cells/well) and incubated at 37 °C in an atmosphere of 5% CO_2. For further cytotoxicity assays, an IC_{50} curve was established; therefore, different dilutions of ethanolic extract (from 100 to 600 μg/mL) and Trolox (from 0.125 to 0.5 mg/mL) were tested in four replicates in two independent experiments. Caco-2 cells were used as an absolute control, and ethanol as a negative control. After incubation with the extract and Trolox for 24 and 48 h, 100 μL of MTT solution (5 mg/mL) was added. After 2 h incubation, 100 μL of stop solution was added to inhibit mitochondrial activity, and the absorbances were measured at 570 nm with a reference at

655 nm (spectrophotometer Tecan i-control, 1.10.4.0 infinite 200Pro), according to the manufacturer's specifications [73]. The absorbance of the treated cultures was subtracted from the absorbance of the untreated cultures and divided by the absorbance of the untreated cultures. The results were multiplied by 100 to calculate the inhibition percent of the viable cells. To calculate the percentage of cytotoxicity, the following equation was used:

$$\% \text{ Inhibition} = (100 \times (\text{Control cells} - \text{Sample cells}))$$

Then the inhibition percent was used to obtain a scatter plot against the concentrations of the extracts and the linear curve were used in the calculation of the IC_{50} for the tested AcE and Trolox.

4.4.3. *A. clematitis* Ethanolic Extract and Trolox Effects on Caco-2 Cells by ABTS Method

Ethanolic plant extracts and Trolox effects were tested on Caco-2 cells alone or in combinations after a concentration corresponding to the IC_{50} calculated by the MTT assay was established. Caco-2 cells were cultivated (1×10^6 cells/well) in six-well plates, where compounds were tested in two groups:

- Group 1: cells were exposed to Trolox (2 mM) and ethanolic extract (AcE) (150 μM) for 24 h;
- Group 2: cells were exposed to ethanol for 24 h; also, Caco-2 cells without compounds were used as absolute control.

After exposure of the cells to the compounds, cells were trypsinized, and split for the ABTS experiments. Cells were washed three times in PBS (pH 7.4), centrifuged at 1000 rpm for 10 min, and the supernatant discarded. The pellet was resuspended in 100 μL PBS (pH 7.4), vortexed for homogenization, sonicated on ice, and centrifuged at 10,000 rpm for 15 min at 4 °C. One hundred μL of cell supernatant was mixed with 200 μL ABTS working solution and left to react at RT for 30 min, and then plated on 96 transparent well plates to read the absorbances at 734 nm. Also, a calibration curve with Trolox concentrations ranging between 0.25 mg/mL and 31 μg/mL was made to calculate the concentrations of cell samples, based on the formula below:

$$\text{T-AOC} = (A_{734}\text{-b}) \div \text{a} \div \text{No. of cells} \times \text{f}$$

Y—$A_{control}$–A_{Trolox}; x—concentration of standard; a—slope of standard curve; b—intercept of standard curve; A_{734}—$A_{control}$–A_{AcE}; f—dilution factor of sample before test; and no. of cells—number of Caco-2 cells in sample per mL of PBS.

The results for the tested Trolox and AcE concentrations were generated from four replicates from two independent experiments.

4.5. Statistical Analysis

The statistical analyses were performed using the Microsoft Office-Excel 2010 Data Analysis Tool (Microsoft Corporation, Redmond, WA, USA) package. The results were calculated and presented as average ± standard deviation (SD), and significant differences were mentioned ($p < 0.05$).

5. Conclusions

The study carried out on the *A. clematitis* L. leaf extracts obtained by maceration in ethanol at room temperature, a formulation similar to the traditional one used in ethnomedicine, revealed for the first time the antioxidant activities of such extracts, which are correlated with their cytotoxic effects on a human colon cancer cell line. Antioxidant activity values obtained from the three types of chemical-based assays calculated with Trolox showed a weak response of the extract (>200 μg/mL) by DPPH and TAC, but moderate response (<150 μg/mL) by the ABTS assay. The total phenolic content showed a low antioxidant activity based on polyphenols, but a moderate activity on total flavonoid content

based on a high level in flavonoids. To our knowledge, these results are obtained for the first time for this type of extract and they demonstrat a moderate antioxidant activity with the cell-based ABTS assay. Also, the ABTS assay used to test the antioxidant activity in the tested cells showed similar effects between the extracts and Trolox. The IC_{50} values obtained by the MTT cytotoxicity assay represent a weak response of the extract on the Caco-2 cells at two timeframes. Therefore, the extract in this formulation used in traditional medicine could be cytotoxic at higher concentrations and after a longer period of time. *A. clematitis* extracts may represent a curative option and a source of low-cost antioxidants, provided short-term use and proper dosage, which could be included into modern healthcare practices. These chemical-based antioxidant assays are low cost and easy to perform and can be used as screening methods of natural products by the scientific or medical community, prior to their chemical characterization.

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References

1. Ang, L.P.; Ng, P.W.; Lean, Y.L.; Kotra, V.; Kifli, N.; Goh, H.P.; Lee, K.S.; Sarker, M.M.R.; Al-Worafi, Y.M.; Ming, L.C. Herbal Products Containing Aristolochic Acids: A Call to Revisit the Context of Safety. *J. Herb. Med.* **2021**, *28*, 100447. [CrossRef]
2. Michl, J.; Jennings, H.M.; Kite, G.C.; Ingrouille, M.J.; Simmonds, M.S.J.; Heinrich, M. Is Aristolochic Acid Nephropathy a Widespread Problem in Developing Countries? *J. Ethnopharmacol.* **2013**, *149*, 235–244. [CrossRef] [PubMed]
3. Kiliś-Pstrusińska, K.; Wiela-Hojeńska, A. Nephrotoxicity of Herbal Products in Europe—A Review of an Underestimated Problem. *Int. J. Mol. Sci.* **2021**, *22*, 4132. [CrossRef] [PubMed]
4. Scarborough, J. Ancient Medicinal Use of *Aristolochia*: Birthwort's Tradition and Toxicity. *Pharm. Hist.* **2011**, *53*, 3–21.
5. Kuo, P.-C.; Li, Y.-C.; Wu, T.-S. Chemical Constituents and Pharmacology of the *Aristolochia* (馬兜鈴 Mădōu Ling) Species. *J. Tradit. Complement. Med.* **2012**, *2*, 249–266. [CrossRef]
6. Bartha, G.S.; Tóth, G.; Horváth, P.; Kiss, E.; Papp, N.; Kerényi, M. Analysis of Aristolochlic Acids and Evaluation of Antibacterial Activity of *Aristolochia clematitis* L. *BioFut* **2020**, *70*, 323–329. [CrossRef]
7. Heinrich, M.; Chan, J.; Wanke, S.; Neinhuis, C.; Simmonds, M.S.J. Local Uses of *Aristolochia* Species and Content of Nephrotoxic Aristolochic Acid 1 and 2—A Global Assessment Based on Bibliographic Sources. *J. Ethnopharmacol.* **2009**, *125*, 108–144. [CrossRef]
8. Michl, J.; Ingrouille, M.J.; Simmonds, M.S.J.; Heinrich, M. Naturally Occurring Aristolochic Acid Analogues and Their Toxicities. *Nat. Prod. Rep.* **2014**, *31*, 676. [CrossRef]
9. Chen, C.H.; Dickman, K.G.; Huang, C.Y.; Moriya, M.; Shun, C.T.; Tai, H.C.; Huang, K.H.; Wang, S.M.; Lee, Y.J.; Grollman, A.P.; et al. Aristolochic Acid-Induced Upper Tract Urothelial Carcinoma in Taiwan: Clinical Characteristics and Outcomes. *Int. J. Cancer* **2013**, *133*, 14–20. [CrossRef]
10. Dickman, K.G.; Chen, C.-H.; Grollman, A.P.; Pu, Y.-S. Aristolochic Acid-Containing Chinese Herbal Medicine and Upper Urinary Tract Urothelial Carcinoma in Taiwan: A Narrative Review. *World J. Urol.* **2022**, *41*, 899–907. [CrossRef]
11. Chen, I.-H.; Luo, H.-L.; Su, Y.-L.; Huang, C.-C.; Chiang, P.-H.; Yu, C.-C.; Lee, N.-L.; Lin, J.-J.; Sung, M.-T. Aristolochic Acid Affects Upper Tract Urothelial Cancer Behavior through the MAPK Pathway. *Molecules* **2019**, *24*, 3707. [CrossRef] [PubMed]
12. Nortier, J.L. Invasive Urothelial Carcinoma after Exposure to Chinese Herbal Medicine Containing Aristolochic Acid May Occur without Severe Renal Failure. *Nephrol. Dial. Transplant.* **2003**, *18*, 426–428. [CrossRef] [PubMed]
13. Jadot, I.; Declèves, A.-E.; Nortier, J.; Caron, N. An Integrated View of Aristolochic Acid Nephropathy: Update of the Literature. *Int. J. Mol. Sci.* **2017**, *18*, 297. [CrossRef] [PubMed]
14. Arlt, V.M.; Stiborova, M.; Vom Brocke, J.; Simoes, M.L.; Lord, G.M.; Nortier, J.L.; Hollstein, M.; Phillips, D.H.; Schmeiser, H.H. Aristolochic Acid Mutagenesis: Molecular Clues to the Aetiology of Balkan Endemic Nephropathy-Associated Urothelial Cancer. *Carcinogenesis* **2007**, *28*, 2253–2261. [CrossRef] [PubMed]

15. Anger, E.E.; Yu, F.; Li, J. Aristolochic Acid-Induced Nephrotoxicity: Molecular Mechanisms and Potential Protective Approaches. *Int. J. Mol. Sci.* **2020**, *21*, 1157. [CrossRef]
16. Arlt, V.M.; Ferluga, D.; Stiborova, M.; Pfohl-Leszkowicz, A.; Vukelic, M.; Ceovic, S.; Schmeiser, H.H.; Cosyns, J.-P. Is Aristolochic Acid a Risk Factor for Balkan Endemic Nephropathy-Associated Urothelial Cancer? *Int. J. Cancer* **2002**, *101*, 500–502. [CrossRef]
17. International Agency for Research on Cancer. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 100 D, Radiation: This Publication Represents the Views and Expert Opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Which Met in Lyon, 2–9 June 2009*; IARC: Lyon, France, 2012; ISBN 978-92-832-1321-5.
18. Grollman, A.P.; Marcus, D.M. Global Hazards of Herbal Remedies: Lessons from *Aristolochia*: The Lesson from the Health Hazards of *Aristolochia* Should Lead to More Research into the Safety and Efficacy of Medicinal Plants. *EMBO Rep.* **2016**, *17*, 619–625. [CrossRef]
19. Akindele, A.J.; Wani, Z.; Mahajan, G.; Sharma, S.; Aigbe, F.R.; Satti, N.; Adeyemi, O.O.; Mondhe, D.M. Anticancer Activity of *Aristolochia ringens* Vahl. (*Aristolochiaceae*). *J. Tradit. Complement. Med.* **2015**, *5*, 35–41. [CrossRef]
20. Alok, S.; Jain, S.K.; Verma, A.; Kumar, M.; Mahor, A.; Sabharwal, M. Herbal Antioxidant in Clinical Practice: A Review. *Asian Pac. J. Trop. Biomed.* **2014**, *4*, 78–84. [CrossRef]
21. Sun, J.; Pan, J.; Liu, Q.; Cheng, J.; Tang, Q.; Ji, Y.; Cheng, K.; Wang, R.; Liu, L.; Wang, D.; et al. Melatonin Attenuates Mitochondrial Damage in Aristolochic Acid-Induced Acute Kidney Injury. *Biomol. Ther.* **2023**, *31*, 97–107. [CrossRef]
22. Efferth, T.; Oesch, F. Repurposing of Plant Alkaloids for Cancer Therapy: Pharmacology and Toxicology. *Semin. Cancer Biol.* **2021**, *68*, 143–163. [CrossRef] [PubMed]
23. Lerma-Herrera, M.A.; Beiza-Granados, L.; Ochoa-Zarzosa, A.; López-Meza, J.E.; Navarro-Santos, P.; Herrera-Bucio, R.; Aviña-Verduzco, J.; García-Gutiérrez, H.A. Biological Activities of Organic Extracts of the Genus *Aristolochia*: A Review from 2005 to 2021. *Molecules* **2022**, *27*, 3937. [CrossRef] [PubMed]
24. Košťálová, D.; Hrochová, V.; Pronayová, N.; Leško, J. Constituents of *Aristolochia clematitis* L. *Chem. Papers* **1991**, *45*, 713–716.
25. Wu, T.-S.; Chan, Y.-Y.; Leu, Y.-L.; Chen, Z.-T. Sesquiterpene Esters of Aristolochic Acid from the Root and Stem of *Aristolochia Heterophylla*. *J. Nat. Prod.* **1999**, *62*, 415–418. [CrossRef] [PubMed]
26. Wu, T.-S.; Damu, A.G.; Su, C.-R.; Kuo, P.-C. Terpenoids of *Aristolochia* and Their Biological Activities. *Nat. Prod. Rep.* **2004**, *21*, 594. [CrossRef]
27. Mariyammal, V.; Sathiageetha, V.; Amalraj, S.; Gurav, S.S.; Amiri-Ardekani, E.; Jeeva, S.; Ayyanar, M. Chemical Profiling of *Aristolochia tagala* Cham. Leaf Extracts by GC-MS Analysis and Evaluation of Its Antibacterial Activity. *J. Indian Chem. Soc.* **2023**, *100*, 100807. [CrossRef]
28. Podea, R.; Culea, M.; Fromondi, L. *The Determination of the Therapeutic Compounds from Aristolochia clematitis by GC/MS*; Studia Universitatis Babeş-Bolyai, Physica: Cluj-Napoca, Romania, 2001; pp. 378–384.
29. Benmehdi, H.; Behilil, A.; Memmou, F.; Amrouche, A. Free Radical Scavenging Activity, Kinetic Behaviour and Phytochemical Constituents of *Aristolochia clematitis* L. Roots. *Arab. J. Chem.* **2017**, *10*, S1402–S1408. [CrossRef]
30. Egbujor, M.C.; Egu, S.A.; Okonkwo, V.I.; Jacob, A.D.; Egwuatu, P.I.; Amasiatu, I.S. Antioxidant Drug Design: Historical and Recent Developments. *J. Pharm. Res. Int.* **2021**, *32*, 36–56. [CrossRef]
31. Xu, D.-P.; Li, Y.; Meng, X.; Zhou, T.; Zhou, Y.; Zheng, J.; Zhang, J.-J.; Li, H.-B. Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources. *Int. J. Mol. Sci.* **2017**, *18*, 96. [CrossRef]
32. Sreeharsha, N.; Kathiravan, G.; Rajagopal, K.; Rajasekar, A.; Rajangam, B.; Aldhubiab, B.; Narayanaswamy, V.; Attimarad, M.; Nair, A.; Karnathi, R.; et al. Synthesis, Characterization, and Biological Activity of Silver Nanoparticles Synthesized from *Aristolochia bracteolata* Lam. *Pharmacogn. Mag.* **2020**, *16*, 568. [CrossRef]
33. Papuc, C.; Crivineanu, M.; Goran, G.; Nicorescu, V.; Durdun, N. Free Radicals Scavenging and Antioxidant Activity of European Mistletoe (*Viscum album*) and European Birthwort (*Aristolochia clematitis*). *Rev. Chim.* **2010**, *61*, 619–622.
34. Barba-Ostria, C.; Carrera-Pacheco, S.E.; Gonzalez-Pastor, R.; Heredia-Moya, J.; Mayorga-Ramos, A.; Rodríguez-Pólit, C.; Zúñiga-Miranda, J.; Arias-Almeida, B.; Guamán, L.P. Evaluation of Biological Activity of Natural Compounds: Current Trends and Methods. *Molecules* **2022**, *27*, 4490. [CrossRef] [PubMed]
35. Mendonça, J.D.S.; Guimarães, R.D.C.A.; Zorgetto-Pinheiro, V.A.; Fernandes, C.D.P.; Marcelino, G.; Bogo, D.; Freitas, K.D.C.; Hiane, P.A.; De Pádua Melo, E.S.; Vilela, M.L.B.; et al. Natural Antioxidant Evaluation: A Review of Detection Methods. *Molecules* **2022**, *27*, 3563. [CrossRef] [PubMed]
36. Christodoulakis, N.S.; Kotsironi, K.; Tsafantakis, N.; Stefi, A.L.; Fokialakis, N. Leaf Structure and Phytochemical Analysis of *Aristolochia baetica*, a Traditionally Used Pharmaceutical Plant. *J. Herbs Spices Med. Plants* **2019**, *25*, 88–103. [CrossRef]
37. Kimura, O.; Haraguchi, K.; Ohta, C.; Koga, N.; Kato, Y.; Endo, T. Uptake of Aristolochic Acid I into Caco-2 Cells by Monocarboxylic Acid Transporters. *Biol. Pharm. Bull.* **2014**, *37*, 1475–1479. [CrossRef]
38. Aiello, P.; Sharghi, M.; Mansourkhani, S.M.; Ardekan, A.P.; Jouybari, L.; Daraei, N.; Peiro, K.; Mohamadian, S.; Rezaei, M.; Heidari, M.; et al. Medicinal Plants in the Prevention and Treatment of Colon Cancer. *Oxidative Med. Cell. Longev.* **2019**, *2019*, 2075614. [CrossRef]
39. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT—Food Sci. Technol.* **1995**, *28*, 25–30. [CrossRef]

40. Ozgen, M.; Reese, R.N.; Tulio, A.Z.; Scheerens, J.C.; Miller, A.R. Modified 2,2-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) Method to Measure Antioxidant Capacity of Selected Small Fruits and Comparison to Ferric Reducing Antioxidant Power (FRAP) and 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) Methods. *J. Agric. Food Chem.* **2006**, *54*, 1151–1157. [CrossRef]
41. Prieto, P.; Pineda, M.; Aguilar, M. Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E. *Anal. Biochem.* **1999**, *269*, 337–341. [CrossRef]
42. Al-Busafi, S.; Al-Harthi, M.; Al-Sabahi, B. Isolation of Aristolochic Acids from *Aristolochia bracteolata* and Studies of Their Antioxidant Activities. *SQU J. Sci.* **2004**, *9*, 19. [CrossRef]
43. Phongpaichit, S.; Nikom, J.; Rungjindamai, N.; Sakayaroj, J.; Hutadilok-Towatana, N.; Rukachaisirikul, V.; Kirtikara, K. Biological Activities of Extracts from Endophytic Fungi Isolated from *Garcinia* Plants: Biological Activities of Extracts from Endophytic Fungi. *FEMS Immunol. Med. Microbiol.* **2007**, *51*, 517–525. [CrossRef] [PubMed]
44. Gyurászová, M.; Gurecká, R.; Bábíčková, J.; Tóthová, Ľ. Oxidative Stress in the Pathophysiology of Kidney Disease: Implications for Noninvasive Monitoring and Identification of Biomarkers. *Oxidative Med. Cell. Longev.* **2020**, *2020*, 5478708. [CrossRef] [PubMed]
45. López-Alarcón, C.; Denicola, A. Evaluating the Antioxidant Capacity of Natural Products: A Review on Chemical and Cellular-Based Assays. *Anal. Chim. Acta* **2013**, *763*, 1–10. [CrossRef] [PubMed]
46. Crivineanu, M.; Durdun, C.; Nicorescu, I. Antioxidant Activity of Some Polyphenolic Extracts Obtained from Plants with Antitumoral Potential on Linoleic Acid Emulsion. *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca Vet. Med.* **2009**, *66*, 359–365. [CrossRef]
47. Bourhia, M.; Laasri, F.E.; Moussa, S.I.; Ullah, R.; Bari, A.; Saeed Ali, S.; Kaoutar, A.; Haj Said, A.A.; El Mzibri, M.; Said, G.; et al. Phytochemistry, Antioxidant Activity, Antiproliferative Effect, and Acute Toxicity Testing of Two Moroccan *Aristolochia* Species. *Evid.-Based Complement. Altern. Med.* **2019**, *2019*, 9710876. [CrossRef]
48. El Omari, N.; Sayah, K.; Fettach, S.; El Blidi, O.; Bouyahya, A.; Faouzi, M.E.A.; Kamal, R.; Barkiyou, M. Evaluation of In Vitro Antioxidant and Antidiabetic Activities of *Aristolochia longa* Extracts. *Evid.-Based Complement. Altern. Med.* **2019**, *2019*, 7384735. [CrossRef]
49. Olugbami, J.O.; Gbadegesin, M.A.; Odunola, O.A. In Vitro Evaluation of the Antioxidant Potential, Phenolic and Flavonoid Contents of the Stem Bark Ethanol Extract of *Anogeissus leiocarpus*. *Afr. J. Med. Med. Sci.* **2014**, *43* (Suppl. S1), 101–109.
50. Jegadeeswari, P.; Daffodil, E.D.; Mohan, V.R. Quantification of Total Phenolics, Flavonoid and in Vitro Antioxidant Activity of *Aristolochia bracteata* Retz. *Int. J. Pharm. Pharm. Sci.* **2014**, *6*, 747–752.
51. Jianu, C.; Goleț, I.; Stoin, D.; Cocan, I.; Lukinich-Gruia, A.T. Antioxidant Activity of *Pastinaca sativa* L. Ssp. Sylvestris [Mill.] Rouy and Camus Essential Oil. *Molecules* **2020**, *25*, 869. [CrossRef]
52. Abbouyi, A.E.; Soukaina, E.M.; Filali-Ansari, N.; Khyari, S.E. Antioxidant effect of extract of rhizomes from Aristolochia clematitis. *J. Chem. Biol. Phys. Sci. (JCBPS)* **2016**, *6*, 427–437.
53. Hadem, K.H.; Sharan, R.; Kma, L. Phytochemicals of *Aristolochia tagala* and *Curcuma caesia* Exert Anticancer Effect by Tumor Necrosis Factor-α-Mediated Decrease in Nuclear Factor kappaB Binding Activity. *J. Basic Clin. Pharma* **2016**, *7*, 1. [CrossRef]
54. Saeed, N.; Khan, M.R.; Shabbir, M. Antioxidant Activity, Total Phenolic and Total Flavonoid Contents of Whole Plant Extracts *Torilis leptophylla* L. *BMC Complement. Altern. Med.* **2012**, *12*, 221. [CrossRef] [PubMed]
55. Nagendra Prasad, K.; Yang, B.; Yang, S.; Chen, Y.; Zhao, M.; Ashraf, M.; Jiang, Y. Identification of Phenolic Compounds and Appraisal of Antioxidant and Antityrosinase Activities from Litchi (*Litchi sinensis* Sonn.) Seeds. *Food Chem.* **2009**, *116*, 1–7. [CrossRef]
56. Aleixo, Á.A.; Camargos, V.N.; Santos, A.C.; Andrade, P.; Marjorie, K.; Herrera, S.; Iara, R.; Ribeiro, M.; Magalhães, J.T.; Magalhães, J.; et al. Antibacterial and Cytotoxic Antibacterial Potential of Ethanol Extract and Fractions from *Aristolochia galeata* Mart. Ex Zucc. *J. Med. Plants Res.* **2014**, *8*, 326–330. [CrossRef]
57. Choi, Y.L.; Kim, J.K.; Choi, S.-U.; Min, Y.-K.; Bae, M.-A.; Kim, B.T.; Heo, J.-N. Synthesis of Aristolactam Analogues and Evaluation of Their Antitumor Activity. *Bioorganic Med. Chem. Lett.* **2009**, *19*, 3036–3040. [CrossRef]
58. Truong, L.H.; Cuong, N.H.; Dang, T.H.; Hanh, N.T.M.; Thi, V.L.; Tran Thi Hong, H.; Quang, T.H.; Nguyen, H.D.; Nguyen Xuan, C.; Nguyen Hoai, N.; et al. Cytotoxic Constituents from *Isotrema tadungense*. *J. Asian Nat. Prod. Res.* **2021**, *23*, 491–497. [CrossRef]
59. Mongelli, E.; Pampuro, S.; Coussio, J.; Salomon, H.; Ciccia, G. Cytotoxic and DNA Interaction Activities of Extracts from Medicinal Plants Used in Argentina. *J. Ethnopharmacol.* **2000**, *71*, 145–151. [CrossRef]
60. Yu, J.Q.; Liao, Z.X.; Cai, X.Q.; Lei, J.C.; Zou, G.L. Composition, Antimicrobial Activity and Cytotoxicity of Essential Oils from *Aristolochia mollissima*. *Environ. Toxicol. Pharmacol.* **2007**, *23*, 162–167. [CrossRef]
61. Ruffa, M.J.; Ferraro, G.; Wagner, M.L.; Calcagno, M.L.; Campos, R.H.; Cavallaro, L. Cytotoxic Effect of Argentine Medicinal Plant Extracts on Human Hepatocellular Carcinoma Cell Line. *J. Ethnopharmacol.* **2002**, *79*, 335–339. [CrossRef]
62. Benarba, B.; Aoues, A.; Vazquez, A.; Ambroise, G.; Meddah, B. *Aristolochia longa* Aqueous Extract Triggers the Mitochondrial Pathway of Apoptosis in BL41 Burkitt's Lymphoma Cells. *Int. J. Green Pharm.* **2012**, *6*, 45. [CrossRef]
63. Lerma-Herrera, M.A.; Beiza-Granados, L.; Ochoa-Zarzosa, A.; López-Meza, J.E.; Hernández-Hernández, J.D.; Aviña-Verduzco, J.; García-Gutiérrez, H.A. In Vitro Cytotoxic Potential of Extracts from *Aristolochia* Foetida Kunth against MCF-7 and bMECs Cell Lines. *Saudi J. Biol. Sci.* **2021**, *28*, 7082–7089. [CrossRef] [PubMed]

64. Wan, H.; Liu, D.; Yu, X.; Sun, H.; Li, Y. A Caco-2 Cell-Based Quantitative Antioxidant Activity Assay for Antioxidants. *Food Chem.* **2015**, *175*, 601–608. [CrossRef] [PubMed]
65. Wu, T.-K.; Wei, C.-W.; Pan, Y.-R.; Cherng, S.-H.; Chang, W.-J.; Wang, H.-F.; Yu, Y.-L. Vitamin C Attenuates the Toxic Effect of Aristolochic Acid on Renal Tubular Cells via Decreasing Oxidative Stress-Mediated Cell Death Pathways. *Mol. Med. Rep.* **2015**, *12*, 6086–6092. [CrossRef] [PubMed]
66. Li, C.; Wang, M.-H. *Aristolochia debilis* Sieb. et Zucc. Induces Apoptosis and Reactive Oxygen Species in the HT-29 Human Colon Cancer Cell Line. *Cancer Biother. Radiopharm.* **2013**, *28*, 717–724. [CrossRef]
67. Siva, C.; Iyappan, M.; Kumar, M.S.; Mary, R.R. Green Synthesis of Silver Nanoparticles by Using *Aristolochia indica* Leaf Extract. *Indian J. Appl. Res.* **2011**, *4*, 542–543. [CrossRef]
68. Thirumagal, D.J. A Study on Silver Nano Particle Production from *Aristolochia bracteata* and Its Antimicrobial Activity. *Int. J. Adv. Res. Dev.* **2018**, *3*, 40–48.
69. NatMed Pro—Search Results. Available online: https://naturalmedicines.therapeuticresearch.com/search.aspx?q=aristolochia+clematitis&go.x=0&go.y=0 (accessed on 1 July 2024).
70. Swain, T.; Hillis, W.E. The Phenolic Constituents of *Prunus domestica*. I.—The Quantitative Analysis of Phenolic Constituents. *J. Sci. Food Agric.* **1959**, *10*, 63–68. [CrossRef]
71. Sánchez-Rangel, J.C.; Benavides, J.; Heredia, J.B.; Cisneros-Zevallos, L.; Jacobo-Velázquez, D.A. The Folin–Ciocalteu Assay Revisited: Improvement of Its Specificity for Total Phenolic Content Determination. *Anal. Methods* **2013**, *5*, 5990. [CrossRef]
72. Shraim, A.M.; Ahmed, T.A.; Rahman, M.M.; Hijji, Y.M. Determination of Total Flavonoid Content by Aluminum Chloride Assay: A Critical Evaluation. *LWT* **2021**, *150*, 111932. [CrossRef]
73. Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [CrossRef]

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Rapeseed—An Important Oleaginous Plant in the Oil Industry and the Resulting Meal a Valuable Source of Bioactive Compounds

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Abstract: Rapeseeds (*Brassica napus*), cultivated widely as a source of oil, generate substantial by-products after oil extraction. Unfortunately, rapeseed meal is considered a waste product and as such is discharged into environment as compost or used as animal feed. However, this meal is rich in bioactive compounds (proteins, minerals, fibers and polyphenols), indicating its potential for the development of value-added products. The meal shows a higher content of minerals, total dietary fibers and proteins. Rapeseed meal contains a proportion of oil rich in polyunsaturated fatty acids, predominately linoleic and α-linolenic acid. The amino acid proportion in the meal is higher than that in the seeds and contains essential amino acids, predominately valine. The analyses show the presence of valuable components in the cake, which makes it suitable for use in obtaining value-added products.

Keywords: circular economy; rapeseed meal; bioactive compounds; nutritive characteristics

1. Introduction

Nowadays, consumers are requesting functional foods rich in nutrients and bioactive compounds with beneficial health effects [1]. Moreover, the growing human population leads to the necessity for sustainable food production [2]. Thus, by-products and residue resulting from the food industry that still contain high nutritive value are valorized and incorporated into the industrial chain, ensuring the circular economy principles [3].

Oilseeds are crop plants whose edible oil is suitable for human consumption. After soybean, rapeseed is the second most cultivated crop, surpassing peanut, sunflower and cottonseed [4]. These seeds possess advantages such as easy cultivation, good environmental sustainability and rich nutritional qualities [5]. Rapeseed is cultivated the most in Germany and France, followed by Russia, Pakistan, Canada, Australia, China and India [6,7].

Rapeseed meal is the biomass that remains after oil extraction. The oil can be extracted by two traditional methods: by using a solvent or a mechanical press (hot or cold pressing) [8]. These by-products are used primarily as animal feed, but they contain bioactive compounds with numerous health benefits for humans (anti-tumoral, -viral, -bacterial and -mutagenic abilities) [9]. Their nutritional composition depends on the extraction method, variety and the growing conditions of seeds [10].

The application of rapeseed meal is limited by its content in anti-nutritional factors, such as glucosinolates, sinapine and its derivates. These factors can be treated with physical, chemical (through the use of ethanol, methanol or acetone), biological (fermentation) and crop breeding methods [11]. The simplest method for decreasing the content of glucosinolates by up to 94% in rapeseed meal is its immersion in water (1:6) for 15–20 min. This treatment also improves the flavor and palatability of the meal [8].

Possibilities for valorizing the meal have been presented in the literature. The conventional methods include incineration, using it as animal feed, composting and biofuel conversion [12]. Increased awareness of environmental issues has led to new valorization methods, namely recovery of valuable components to create new value-added products [13,14] and the production of functional ingredients [10], new products and biopolymer films [15].

Meal is potentially a source of phenolic compounds [16]. Target compounds can be isolated by ultrasound-assisted extraction (UAE), a method that uses acoustic energy and an extraction solvent [17]. Ultrasound waves induce disruption of the cell walls (according to a dynamic process called cavitation) that facilitates the transfer of some compounds into the extraction medium. Moreover, a localized heating effect enhances the extraction process [5].

The objective of this study was focused on a nutritional, functional and safety assessment of rapeseeds, as well as the resulting meal after oil cold pressing extraction. The efficiency of different ultrasound-assisted treatments and solvents in terms of the total phenolic content and antioxidant activity of the rapeseed meal was also investigated (Figure 1).

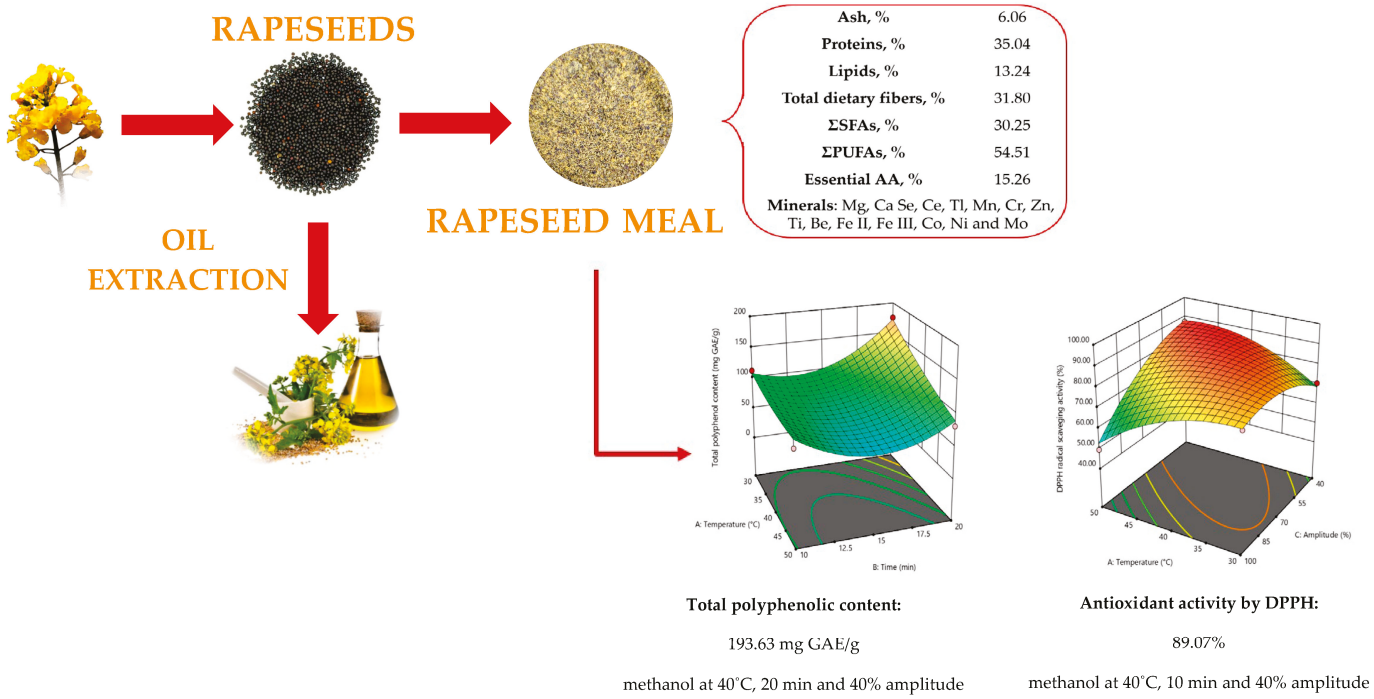


Figure 1. Rapeseeds and meal.

2. Materials and Methods

2.1. Sample Description

Rapeseeds (RSs), rapeseed meal (RSOC) and rapeseed oil (RO) were purchased from an oil factory OLEOMET, in Romania. The seeds were manually cleaned to separate foreign materials, while the cake was ground and sieved (Retsch Vibratory Sieve Shaker AS 200 basic, Haan, Germany) to below 400 µm.

2.2. Raw Material Safety

The safety of the cake was demonstrated by the following analyses: water activity, spectroscopic methods and the ELISA method.

The water activity index (aw) was measured using an AquaLab 4TE water activity meter (Meter Group, Pullman, WA, USA).

The ELISA (enzyme-linked immunosorbent assay) method was performed using kits provided by ProGnosis Biotech S.A. (Larissa, Greece). The samples were analyzed for their content of zearalenone, ochratoxin A, aflatoxin B1 and deoxynivalenol [18].

Mineral elements were determined using ICP-MS (Agilent Technologies 7500 Series, Santa Clara, CA, USA) to highlight possible heavy metal contamination of the samples.

2.3. Nutritive Composition

The seeds and meal were investigated for their moisture, protein, ash, lipid and total dietary fiber content.

Their moisture was determined with a gravimetric method (ISO 665:2020 [19], AOAC935.29 [20]) by drying (at 105 °C) the samples in a laboratory oven (Zhicheng Analysis Instruments, Shanghai, China) until they reached a constant mass.

Protein content was determined by the Kjeldahl method (AOAC 950.48 [21]) with a conversion factor of 5.88.

The ash was determined by calcining 5 g of a sample at 550 °C for 6 h according to AOAC method 923.03 [22].

The fat content was determined using an automated Soxhlet extraction system with petroleum ether as the solvent (ISO 659:2020 [23], AOAC 920.39 [24]).

The total dietary fiber was determined according to AOAC 985.29 [25] using the Megazyme assay kit (Megazyme, Wicklow, Ireland).

The carbohydrate and energy values were calculating by applying Equations (1) and (2):

$$\text{Carbohydrates (\%)} = 100 - (\text{moisture} + \text{ash} + \text{protein} + \text{fat} + \text{fiber}) \quad (1)$$

$$\text{Energy value (Kcal/100 g)} = (4 \times \text{protein}) + (9 \times \text{fat}) + (4 \times \text{carbohydrates}) + (2 \times \text{fibers}) \quad (2)$$

2.4. Physical Properties of the Rapeseeds

2.4.1. Shape and Spatial Dimensions

Five randomized groups of whole seeds (100 pieces) were weighed to an accuracy of 0.1 mg using an analytical balance (PARTEN AS 220.R2, Radwag, Bucharest, Romania).

The seeds' dimensions (length—L; width—W; thickness—T) were measured using a digital caliper (VOREL 15240, Wrocław, Poland, 0.003 mm accuracy).

The geometric mean diameter (D_g), sphericity (ψ), surface area (S), projected area (A_p) and volume (V) of the seeds were determined through comparison to a sphere using the following relationships [2]:

$$D_g(\text{mm}) = \sqrt[3]{L \times W \times T} \quad (3)$$

$$\psi = Dg/L \quad (4)$$

$$S\ (\text{mm}^2) = \Pi \times Dg^2 \quad (5)$$

$$A_p\ \left(\text{mm}^2\right) = \ \times\ L \times W \quad (6)$$

$$V\ (\text{mm}^3) = W \times L \times T \times \varphi \quad (7)$$

2.4.2. Gravimetric Properties

Bulk density (p_b) was measured by filling a container with a 250 mL (V) capacity from a falling height of 150 mm and weighing the content (M), while the true density (p_t) was determined using a pycnometer by recording the volume of toluene (M_T; $p_{toluene}$ = 0.867 g/mL) displaced after the immersion of a known quantity of seeds (M_s) [26].

$$p_b\ (\text{kg/m}^3) = M/V \quad (8)$$

$$p_t\ (\text{kg/m}^3) = (M_s \times p_{toluene})/M_T \quad (9)$$

Porosity (Φ) was calculated according to Equation (10):

$$\Phi\ (\%) = ((p_t - p_b)/p_t) \times 100 \quad (10)$$

2.5. Color and Functional Properties of the Meal

The color of the press cakes was measured using a CR-400 colorimeter (Konica Minolta, Tokyo, Japan) and the CIELAB system. The L* coordinate measures brightness (0 for black

and 100 for white). A negative value for the a* coordinate indicates intensity of the color green, and a positive value indicates intensity of the color red. Parameter b* varies between −100 (indicating intensity of the color blue) and +100 (indicates intensity of the color yellow).

The water/oil holding capacity (WHC/OHC) was measured according to Omowaye-Taiwo et al. [27] with minor modifications: 1 g of the sample and 10 mL of distilled water/corn oil was placed into centrifuge tubes. These were kept at room temperature for 30 min and then centrifuged at 7000 rpm for 20 min. The results were expressed as grams of water/oil absorbed per gram of the sample.

The bulk density was analyzed using a volumetric method. Thus, 5 g of the sample was placed into a 100 mL cylinder, and it was gently tapped 20 times. Values were calculated as the ratio of the sample weight to the sample volume [28].

Least gelatinization concentration (LGC) was determined according to Marasingheand Rani [29,30] with some modifications. Suspensions of the samples and distilled water from 2% to 20% (w/v) were prepared in centrifuge tubes. The tubes were heated for 1 h in a boiling water bath and then rapidly cooled in water at 4 °C for 3 h. When inverting the tube, the concentration of the sample that does not fall or slide is considered the LGC.

Powder wettability was estimated using the method described by North et al. [31]. In brief, 2 grams of cake was transferred into a beaker containing 80 mL of distilled water. The behavior of the powder on the water's surface was observed immediately after its addition. After 30 min of observation, the suspension was stirred fast enough to form a vortex that reached the bottom of the beaker. The contents were agitated for one minute. The degree of wetting was recorded as excellent, good, satisfactory or poor depending on time and the dispersion behavior.

Emulsion capacity (EC) and stability (ES) were analyzed according to the methods proposed by Rani [30] and Iyenagbe et al. [32]: First, 30 mL of a 0.5%suspension was mixed with 10 mL of corn oil. The emulsion was homogenized and immediately transferred into a graduated cylinder (50 mL) to read the height obtained. Emulsion stability was determined by heating the cylinder for 30 min at 80 °C. The final height of the emulsion was read.

$$EC\ (\%) = \frac{Oil\ layer\ height}{Total\ suspension\ height} \times 100 \tag{11}$$

$$ES\ (\%) = \frac{Final\ suspension\ height\ after\ heating}{Initial\ suspension\ height} \times 100 \tag{12}$$

Foam capacity (FC) and stability (FS) were determined using the method proposed by Naczk et al. [33], with some modifications. A suspension of 3 g of meal and 100 mL of distilled water was homogenized for 5 min at 1600 rpm. The mixture was immediately transferred into a 250 mL graduated cylinder, and the foam volume was noted. FS was determined by the decrease in the foam volume as a function of time (20, 40, 60 and 120 min). The results were calculated using the following equations:

$$FC\ (\%) = \frac{volume\ after\ agitation - volume\ before\ agitation}{volume\ before\ agitation} \times 100 \tag{13}$$

$$FS\ (\%) = \frac{foam\ volume\ after\ a\ set\ time}{initial\ foam\ volume} \times 100 \tag{14}$$

2.6. Seed and Meal Comparisons

2.6.1. Fatty Acids

The seeds and oleaginous cake were analyzed to highlight the composition in fatty acids (FAMEs). Their derivatization was performed according to the following procedure: 30 mg of the oil sample was mixed with 2 mL of isooctane. The solution was then subjected to transesterification through the addition, under vigorous stirring, of 200 µL of potassium

hydroxide (2 mol/L methanolic solution). The resulting organic phase was mixed with sodium sulfide, and the supernatant was collected [34].

FAME separation was performed using a gas chromatograph (GC-FID, Agilent Technologies, 6890N GC, Wilmington, DE, USA) and using a stationary polyethylene glycol DB-WAX capillary column (30 m length, 0.25 mm inner diameter and 0.25 μm thickness). The initial temperature of the oven was set to 60 °C for 1 min, it was increased from 60 °C to 200 °C (by 10 °C/min) and held for 2 min and then increased again (by 5 °C/min) from 200 °C to 220 °C and held for 20 min. The flow rates were maintained at 40, 450 and 30 mL/min for hydrogen, air and helium respectively. The injection port and the detector temperatures were 250 °C. FAME identification and quantification were performed by comparing their retention times with those of the standard mixture. Fatty acid composition was expressed as μg/mL and as the relative level (%) of fatty acid composition. Each determination was performed three times [35].

2.6.2. Free Amino Acids

A mixture of the sample (0.7 g) and 15% trichloroacetic acid (6 mL, TCA) was made. The mixture's pH was adjusted to 2.2 ± 0.05 with sodium hydroxide solution (1–4 M), and then the volume was brought to 10 mL exactly with acid. The solution was centrifuged for 5 min at 3000 rpm, and the supernatant was filtered (trough 0.45 μm). The solution containing amino acids was analyzed using the EZ:faast GC-MS kit (Phenomenex, Torrance, CA, USA) [36].

The analysis was performed using a gas chromatograph coupled with a mass spectrometer (Shimadzu, Kyoto, Japan) and a ZB-AAA column(10 m × 0.25 mm). The analysis time was 10 min, and the injected volume was set to 0.002 mL. The initial temperature of the oven was 110 °C, which was then increased to 320 °C and held for three minutes. The conditions for the mass spectrometer were 200 °C for the ion source and 320 °C for the interface. The amino acid mixture solutions included in the kit mentioned above were used for calibration [37].

2.6.3. Mineral Content

The ash resulting after calcination was dissolved with 65% nitric acid (0.73 mL), and the solution was brought to a 50 mL volume with deionized water [2]. The minerals (Li, Be, Mg, Ti, Tl, Co, As, Ca, Cd, Cr, Ce, Cu, Hg, Fe, Mn, Ni, Pb, Se, Sr, Sb, Mo, V and Zn) were estimated with an inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies, Santa Clara, CA, USA).

2.7. Fourier Transform Infrared-Attenuated Total Reflectance (FTIR-ATR)

The meal powders were analyzed using FTIR-ATR. Their spectra were recorded using a Nicolet iS20 spectrometer (Thermo Scientific, Karlsruhe, Germany) equipped with an attenuated total reflectance accessory and a diamond crystal. The spectra were collected within the range of 400 cm^{-1} to 4000 cm^{-1} at a 4 cm^{-1} resolution and using 32 scans. The spectra obtained were processed using OMNIC software (version 9, Thermo Scientific) [38].

2.8. Preparation of the Rapeseed Meal Extract

The application of different treatments (in terms of choice of solvent, use of heat, agitation and time) caused a change in the content of total polyphenols (the TPC) and, implicitly, the free radical scavenging activity (DPPH). Evaluation of these changes was carried out using the software Design Expert 11 (Stat-Ease Inc., Minneapolis, MN, USA, trial version) through the response surface methodology using the Box–Behnken model. After studying the literature in the model, 4 factors were varied, and 3 replications were provided at the central point. Each factor was varied at 3 levels (Table 1) as follows: temperature (A: 30, 40 and 50 °C), time (B: 10, 15 and 20 min), amplitude (C: 40, 70 and 100%) and solvent (D: 80% methanol, water and 80% ethanol) [39–41].

Table 1. Experimental values (coded and actual).

Factors	Values		
	−1	**0**	**+1**
Temperature (°C) (A)	30	40	50
Time (min) (B)	10	15	20
Amplitude (%) (C)	40	70	100
Solvent (D1)	Methanol	Ethanol	Water
Solvent (D2)	Methanol	Water	Ethanol

The extracts were made by sonicating a mixture of defatted meal and the solvent (at a ratio of 1:20) in an ultrasonic bath (Elma Transsonic TI-H15, Singen, Germany), varying the parameters of temperature, time and amplitude and keeping the frequency constant at 45 Hz.

Ultrasound energy is the product of frequency and amplitude. If the frequency is constant, the only possibility for changing the ultrasonic energy is changing the amplitude of the sinusoidal signal [42].

2.9. Total Phenolic Content (TPC)

To determine the TPC, 0.2 mL of the extract prepared as described previously was mixed with 2 mL of Folin–Ciocâlteu reagent (diluted 1:10) and 1.8 mL of 7.5% sodium carbonate. The mixture was left at room temperature for 30 min, and the absorbance was read at a 750 nm wavelength using an UV-VIS NIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan) [43]. Gallic acid was used to generate calibration curves at concentrations of 10–500 mg/L. The regression coefficient was 0.99658, while the equation y = 0.00484x + 0.16058. The sample was analyzed in duplicate.

2.10. DPPH Radical Scavenging Activity

For determination of the radical scavenging activity, 0.3 mL of the extract prepared as described previously was mixed with 2.7 mL of 0.1 mM DPPH reagent (prepared in methanol). The mixture was shaken and kept at room temperature for 30 min [44]. The absorbance was read at a 517 nm wavelength using an UV-VIS-NIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The results were expressed as the percentage of DPPH discoloration according to Equation (8):

$$Scavenging\ effect(\%) = \frac{Absorbance_{DPPH} - Absorbance_{sample}}{Absorbance_{DPPH}} \times 100 \quad (15)$$

2.11. Statistical Analysis

All the results are presented as mean ± standard deviation. The nutritional and functional analyses for the rapeseeds and meal were performed in triplicate. Meanwhile, the amino acid, fatty acid and mineral analyses were performed in duplicate. A sample of 500 seeds was used for physical characterization of the seeds. The values obtained were processed using Excel-Stat software (trial version). Differences between the samples were established using an analysis of variance (ANOVA) and using Tukey's test at a 95% confidence level. To determine the chemical differences between the seeds and meal, Student's *t*-test was performed.

3. Results and Discussion

3.1. Meal Safety

The value obtained for water activity was low (0.4153), lower than 0.6, which does not allow for the development of molds, yeasts or bacteria.

The mineral analysis using ICP-MS showed the absence of heavy metals such as lead, mercury and cadmium.

The results for the studied mycotoxins fell within the legal stability limit allowed by the European Union, and these are presented in Table 2.

Table 2. Incidence of mycotoxins in rapeseed meal.

Sample	Limit of Detection µg/kg	Limit of Quantification µg/kg	Results µg/kg	Maximum Limit 2006/576/EC µg/kg
Zearalenone	10	15	25.17 ± 2.83	2000
Ochratoxin A	0.5	1.5	3.88 ± 0.62	50
Aflatoxin B_1	0.3	0.7	<LOQ	10
Deoxynivalenol	0.011	0.042	0.207 ± 0.035	0.9

3.2. Physical Properties

The rapeseeds showed variations in their physical properties (Table 3), between 1.70 and 2.88 mm for length; between 1.60 and 2.73 mm for width; between 1.22 and 2.49 mm for thickness; between 1.61 and 2.55 for the geometric mean diameter; between 3.78 and 15.01 mm^3 for volume; and between 8.13 mm^2 and 20.36 mm^2 for the surface area. The average sphericity was 91%, which indicated that the seeds are spherical and easily roll on structural surfaces. The values for bulk density, true density and porosity were 694.20 kg/m^3, 1070.17 kg/m^3 and 37.50%, respectively.

Table 3. Shape, dimensional and gravimetric parameters for the seeds; n = 500.

Sample	Range	Average
Shape and dimensional parameters		
L, mm	1.70–2.88	2.24 ± 0.20
W, mm	1.60–2.73	2.06 ± 0.17
T, mm	1.22–2.49	1.85 ± 0.18
D_g, mm	1.61–2.55	2.04 ± 0.15
Ψ, -	0.75–1.09	0.91 ± 0.05
V, mm^3	3.78–15.01	7.87 ± 1.84
S, mm^2	8.13–20.36	13.14 ± 1.96
A_p, mm^2	2.24–5.79	3.64 ± 0.57
Gravimetric parameters		
M, g	0.0028–0.0099	0.0057 ± 0.001
mass of 1000 grains, g	5.29–5.47	5.37 ± 0.08
p_b, kg/m^3	686.05–698.56	694.20 ± 3.36
p_t, kg/m^3	898.82–1176.68	1070.17 ± 98.57
φ, %	22.81–41.25	37.50 ± 2.33

L, W, T, M, D_g, Ψ, V, S, A_p—length, width, thickness, mass, geometric mean diameter, sphericity, volume, surface area and projected area of sunflower seeds; p_b—bulk density; p_t—true density; φ—porosity.

Compared to the Turan variety, our seeds are longer but have the same thickness and width [38]. The opposite was observed for the varieties Elvis and Capitol, which are longer and narrower, and for the varieties Jetneuf and Samurai, which are longer and larger than those investigated in this study [45,46].

The results found were consistent with the best claims by other authors (L = 1.52–2.96 mm; W = 1.47–2.68 mm; T = 0.99–2.01 mm; D_g = 1.63–2.23 mm; Ψ = 0.82–0.97; mass of 1000 grains = 2.85–6.36 g; S = 1.67–15.7 mm^2; Φ = 1.62–10.07 mm^3; bulk density = 585.1–738.8 kg/m^3; true density = 1091.3 kg/m^3; M = 0.0038–0.0065 g; A_p = 3.60–4.67 mm^2) [45–52].

The correlation coefficients (Table 4) show the relationships between the dimensional properties of the seeds. All correlations found, except D_g/Ψ and Ψ/S, were significant at $p < 0.5\%$. The parameters L, W and T showed moderate positive correlations, indicating

a certain dependence between them (long seeds are also the widest, r = 0.676, and wide seeds are also the thickest, r = 0.543).

Table 4. Correlation between the physical properties of rapeseeds; n = 500.

Variables	M	L	W	T	D_g	Ψ	V	S	A_p
M	1								
L	0.167 *	1							
W	0.088 *	0.676 *	1						
T	0.009	0.365 *	0.543 *	1					
D_g	0.100 *	0.803 *	0.877 *	0.800 *	1				
Ψ	−0.133 *	−0.529 *	0.116 *	0.525 *	0.078	1			
V	0.079	0.654 *	0.867 *	0.871 *	0.971 *	0.284 *	1		
S	0.108 *	0.804 *	0.876 *	0.796 *	0.999 *	0.075	0.975 *	1	
A_p	0.149 *	0.917 *	0.910 *	0.489 *	0.913 *	−0.233 *	0.829 *	0.916 *	1

The symbol * indicates significance at $p < 0.05$; L, W, T, M, Dg, Ψ—length, width, thickness, mass, geometric mean diameter and sphericity of the seeds.

The influence of mass was investigated by calculating the correlation coefficients for all combinations. Although significant values were found at $p < 0.05$, they all indicated weak linear correlations (r = 0.088–0.167), indicating independence between them. Therefore, longer, wider and denser seeds are not necessarily the heaviest.

The physical properties (length, width and thickness) were integrated into the formula for calculating the geometric mean diameter, surface, sphericity and volume. For this reason, strong positive correlations between these formulas were easy to achieve.

3.3. Nutritional Composition of the Seeds and Meal

3.3.1. Chemical Composition

The nutritional composition (Table 5) of the whole seeds and meal after cold pressing extraction presented significant differences ($p < 0.05$). Except for fat content, all the parameters (moisture, ash, proteins, fibers and carbohydrates) were increased in the meal. This may be related to the cold extraction method, which leaves a significant content of oil that increases the nutritional value of the meal. The nutritional values, except for the moisture, were significantly different at $p < 0.05$.

Table 5. Nutritional composition of rapeseeds and meal.

Parameter	Seed	Meal
Moisture, %	5.24 ± 0.13^{a}	5.27 ± 0.12^{a}
Ash, %	4.26 ± 0.02^{b}	6.06 ± 0.03^{a}
Proteins, %	24.29 ± 0.02^{b}	35.04 ± 0.32^{a}
Lipids, %	35.22 ± 0.40^{a}	13.24 ± 0.03^{b}
Total dietary fibers, %	$22.95 \pm 0{,}16^{b}$	$31.80 \pm 0{,}67^{a}$
Remaining carbohydrates, %	8.04 ± 0.43^{b}	8.58 ± 0.78^{a}
Energy value, kcal/100 g	492.24 ± 1.80^{a}	342.32 ± 1.14^{b}

Difference assessment was performed with a paired *t*-test. Values followed by $^{a, b}$ are statistically different at the 95% confidence level.

Various studies on the nutritional properties of rapeseed meal (after cold extraction) have shown that the moisture, ash, proteins, lipids, total dietary fiber and carbohydrates ranged between 6 and 10.8%, 4.19 and 19.7%, 14.03 and 40.1%, 5.14 and 23.1%, 5.5 and 20.11% and 8.35 and 48%, respectively [8].

Regarding the seeds, the nutritional parameters found in the literature vary as follows: moisture: 1.96–6.4%; ash: 3.8–4.84%; proteins: 20.85–25.7%; lipids: 38.80–40.60% [45].

Due to the high fat content (35.22%), which has the highest energetic contribution (9 kcal/g), the seeds have a higher caloric value. Similar values have been found in the literature (559.60–572 kcal/100 g in rapeseeds and 348,2 kcal/100 g in meal [53,54])

3.3.2. Mineral Composition

Oilseeds are a valuable source of mineral elements (Table 6) that have an important role for human health (with involvement in basic biological mechanisms). The major macroelements identified in the rapeseed meal were Mg and Ca, which represented 73.60% of the total ash content (6.06%). Among the microelements, Se showed the highest concentration followed by Ce, Tl, Mn, Cr, Zn, Ti, Be, Fe II, Fe III, Co, Ni and Mo. After oil extraction, except Tl and Li, all the minerals increased. The element Li was the only element not present in the meal.

Table 6. Comparison of mineral composition for rapeseeds, meal and oil.

Mineral Composition	Seed	Meal	Oil
Lithium (Li), mg/kg	1.10 ± 0.02^{a}	-	1.00 ± 0.01^{b}
Beryllium (Be), mg/kg	17.90 ± 1.10^{b}	26.30 ± 0.40^{a}	0.40 ± 0.02^{c}
Magnesium (Mg), mg/kg	4679.4 ± 360.5^{a}	4194.8 ± 0.00^{a}	8.00 ± 0.57^{b}
Calcium (Ca), mg/kg	2139.6 ± 134.5^{b}	3085.6 ± 69.3^{a}	6.70 ± 0.47^{c}
Titan (Ti), mg/kg	23.50 ± 1.90^{a}	26.60 ± 2.50^{a}	0.10 ± 0.00^{b}
Chromium (Cr), mg/kg	73.00 ± 7.10^{b}	108.5 ± 6.3^{a}	1.00 ± 0.04^{c}
Manganese (Mn), mg/kg	73.60 ± 6.70^{b}	120.7 ± 9.3^{a}	0.80 ± 0.10^{c}
Iron (Fe-II), mg/kg	3.60 ± 0.19^{b}	5.60 ± 0.30^{a}	0.03 ± 0.00^{c}
Iron (Fe-III), mg/kg	1.10 ± 0.10^{b}	1.60 ± 0.10^{a}	0.01 ± 0.00^{c}
Cobalt (Co), mg/kg	1.20 ± 0.02^{a}	1.50 ± 0.30^{a}	0.03 ± 0.00^{b}
Nickel (Ni), mg/kg	0.80 ± 0.02^{b}	1.20 ± 0.00^{a}	0.20 ± 0.00^{c}
Copper (Cu), mg/kg	35.60 ± 2.50^{b}	53.20 ± 0.60^{a}	0.60 ± 0.00^{c}
Zinc (Zn), mg/kg	47.40 ± 3.20^{b}	80.80 ± 4.80^{a}	0.20 ± 0.01^{c}
Selenium (Se), mg/kg	1235.1 ± 99.7^{a}	1396.2 ± 92.5^{a}	2.00 ± 0.10^{b}
Molybdenum (Mo), mg/kg	0.30 ± 0.02^{b}	0.40 ± 0.00^{a}	0.20 ± 0.01^{c}
Cesium (Ce), mg/kg	356.9 ± 23.6^{a}	501.3 ± 4.04^{a}	63.7 ± 2.00^{a}
Thallium (Tl), mg/kg	806.2 ± 65.9^{a}	278.2 ± 15.0^{a}	186.6 ± 9.75^{a}
Total, mg/kg	9496.36	9882.71	271.28

Different superscript letters are significantly different ($p < 0.05\%$) according to Tukey's post hoc test.

For the meal, the following values were found in the literature: Cu: 3.42–10.00 mg/kg; Fe: 69.23–159 mg/kg; Mn: 37.77–62.26 mg/kg; Mo: 0–1.5 mg/kg; Zn: 45.00–71.00 mg/kg; Se: 0–1.22 mg/kg; Ca: 5750–7330 mg/kg; Mg: 3500–4690 mg/kg [55,56].

A total of 17 elements were found in the seeds (Mg > Ca > Se > Tl > Ce > Mn > Cr > Zn > Cu > Ti > Be > Fe II > Co > Fe III > Li > Ni > Mo) and oil (Tl > Ce > Mg > C > Se > Li > Mn > Cu > Be > Zn > Mo > Ni > Ti > Fe II > Co > Fe III). In the seeds, the macroelements represented 71.83% of the total ash content (4.26%).

The values found for the seeds were similar to those found by other authors: Ca: 3560–29,955 mg/kg; Mg: 2483.00–3543 mg/kg; Fe: 30.93–195 mg/kg; Mn: 15.56–96.30 mg/kg; Cu: 1.25–21.25 mg/kg; Zn: 21.46–88.90 mg/kg; Cr: 0.80–1.65 mg/kg; Ni: 1.85–2.40 mg/kg [53,57].

A comparative study of the mineral composition showed that the majority of the elements were increased in the meal, and only the content of Tl and Mg was decreased. Li was the only element not found in the meal because it passed into the extracted oil.

In the oil Mg, Ca, Ce and Tl were present to high values, while the other elements were present in proportions $\leq 2\%$. The values found in the literature for rapeseed oil were lower than those found in our study [57].

3.3.3. Fatty Acids

The remaining oil provides its nutritional and health properties to the partially defatted powder, especially in increasing the content of unsaturated fatty acids (Table 7). A total of 31 fatty acids were determined, of which 15 were saturated (SFAs), 7 were monounsaturated (MUFAs) and 9 were polyunsaturated (PUFAs). The difference between the samples was significant at the 95% confidence level.

Table 7. Fatty acid composition of rapeseeds and meal.

Parameter		Seed	Meal
Caprylic acid (C8:0)	SFA	0.41 ± 0.01 [b]	0.65 ± 0.04 [a]
Capric acid (C10:0)	SFA	0.36 ± 0.03 [a]	0.23 ± 0.01 [b]
Lauric acid (C12:0)	SFA	0.37 ± 0.02 [a]	0.21 ± 0.00 [b]
Myristic acid (C14:0)	SFA	0.55 ± 0.04 [a]	0.48 ± 0.03 [a]
Myristoleic acid (C14:1, n-5)	MUFA	0.21 ± 0.01 [b]	1.71 ± 0.06 [a]
Pentadecanoic acid (C15:0)	SFA	4.45 ± 0.28 [a]	5.45 ± 0.35 [a]
cis-10-pentadecanoic acid (C15:1, n-5)	MUFA	6.21 ± 0.03 [a]	-
Palmitic acid (C16:0)	SFA	12.27 ± 0.87 [a]	-
Palmitoleic acid (C16:1, n-7)	MUFA	0.52 ± 0.01 [a]	0.65 ± 0.04 [a]
Heptadecanoic acid (C17:0)	SFA	-	22.17 ± 0.26 [a]
Stearic acid (C18:0)	SFA	1.08 ± 0.03 [a]	0.47 ± 0.02 [b]
Oleici acid + elaidic acid (C18:1, cis + trans, n-9)	MUFA	10.24 ± 0.49 [a]	9.12 ± 0.16 [a]
Linoleic acid + Linolelaidic acid (C18:2, cis + trans, n-6)	PUFA	44.72 ± 0.64 [a]	39.63 ± 0.67 [b]
γ-Linolenic acid (C18:3, n-6)	PUFA	0.64 ± 0.00 [a]	-
α-Linolenic acid (C18:3, n-3)	PUFA	1.17 ± 0.07 [b]	12.48 ± 0.03 [a]
cis-11,14-eicosadienoic acid + cis-8,11,14-eicosatrienoic acid (C20:2, n-6)	PUFA	1.26 ± 0.01 [b]	1.75 ± 0.07 [a]
cis-11,14,17-eicosatrienoic acid (C20:3, n-3)	PUFA	0.31 ± 0.01 [a]	0.40 ± 0.03 [a]
Arachidonic acid (C20:4, n-6)	PUFA	0.46 ± 0.06 [a]	-
Heneicosanoic acid (C21:0)	SFA	0.38 ± 0.01 [a]	0.50 ± 0.04 [a]
Eicosadienoic acid (C22:0)	SFA	0.21 ± 0.01 [a]	0.10 ± 0.00 [b]
Erucic acid (C22:1, n-9)	MUFA	5.57 ± 0.18 [a]	3.75 ± 0.14 [b]
cis-4,7,10,13,16,19-docosahexanoic acid (C22:2, n-6)	PUFA	-	0.25 ± 0.014 [a]
cis-4,7,10,13,16,19-docosa-hexanoic + nervonic acid (C22:6, n-3 + C24:1, n-9)	PUFA	4.31 ± 0.11 [a]	-
Tricosanoic acid (C23:0)	SFA	4.15 ± 0.03 [a]	-
Lignoceric acid (C24:0)	SFA	0.17 ± 0.01 [a]	-
C18:2 w-6/C18:3 w-3		38.22 ± 1.77	3.17 ± 0.06
C18:1 w-9/C18:2 w-6		0.23 ± 0.01	0.23 ± 0.00
Σ*SFAs (%)*		24.40 ± 0.56	30.25 ± 0.64
Σ*UFAs (%)*		75.62 ± 1.22	69.75 ± 0.69
Σ*MUFAs (%)*		22.75 ± 0.35	15.24 ± 0.13
Σ*PUFAs (%)*		52.87 ± 0.87	54.51 ± 0.56
Σ*SFAs*/Σ*UFAs*		0.32 ± 0.01	0.43 ± 0.01
Σ*PUFAs*/Σ*MUFAs*		2.30 ± 0.00	3.60 ± 0.01

Different superscript letters means a significant difference ($p < 0.05\%$).

The seed oil contained 24.40% saturated fatty acids (SFAs), 22.75% monounsaturated fatty acids (MUFAs) and 52.87% polyunsaturated fatty acids (PUFAs). The most predominant fatty acids identified in the seeds were linoleic and linolelaidic acids, followed by palmitic, oleic and elaidic acids. Eicosadienoic and myristoleic acids were found in small amounts. A lower amount of stearic acid was found than palmitic acid.

The rapeseed meal contained 30.25% SFAs, 15.24% MUFAs and 54.51% PUFAs. Compared to the seeds, in the SFA classes of the meal, an absence of palmitic, tricosanoic and lignoceric acids was observed, as was the presence of heptadecanoic acid. Linoleic, linolelaidic, heptadecanoic and α-linolenic acids were the most predominant fatty acids present in the rapeseed meal. Rapeseeds and meal were found to be poorer in SFAs and PUFAs in the literature [53,56,57].

Excessive consumption of saturated fatty acids has a negative effect on human health, especially on the cardiovascular system. In this regard, both investigated samples were rich in UFAs. Moreover, PUFAs were more abundant than MUFAs in both samples, and these findings indicate their importance for health. PUFAs improve fluidity and permeability through cellular membranes and reduce the risk of cardiovascular diseases and autoimmune disorders [35].

PUFAs n-6 and n-3 are essential fatty acids that cannot be produced by the human body and must be taken in our diet [2]. In a healthy balanced diet, the optimal n-6/n-3 ratio ranges between 1:1 and 5:1. However, the modern diet includes high consumption of PUFAs n-6 (at ratios of 10:1 and 20:1), which increases the risk of developing inflammatory diseases, such as obesity [58]. The group of PUFAs n-3 investigated in this study was represented by α-Linolenic acid, cis-11,14,17-eicosatrienoic acid and cis-4,7,10,13,16,19-docosa-hexanoic acid. On the other hand, the group of PUFAs n-6 was represented by γ-Linolenic acid, Linoleic acid + Linolelaidic acid, cis-11,14-eicosadienoic acid + cis-8,11,14-eicosatrienoic acid, arachidonic acid and cis-4,7,10,13,16,19-docosahexanoic acid (C22:2, n-6).

The n-6/n-3 PUFA ratio in the meal was 3.17. This value was lower than that in the seeds (34.29), indicating a positive nutritional profile and a beneficial effect on cardiovascular risk factors [59]. The values found were much lower than those found in the literature, which ranged between 5.46 and 7.08 [55].

3.3.4. Amino Acids

The content of protein in the rapeseed meal was increased the most (35.04% for the meal vs. 24.29% in the seeds). Considering this increase, an evaluation of protein quality by determining the amino acid (AA) profile is necessary (Table 8).

Table 8. Amino acid composition of rapeseeds and meal.

Amino Acids	Seed		Meal	
	nmol/g	%	nmol/g	%
Alanine	2081.39 ± 60.32 ^{b}	5.37	2497.15 ± 58.12 ^{a}	5.46
Glycine	2192.27 ± 119.30 ^{b}	5.65	2886.41 ± 140.32 ^{a}	6.31
α-aminobutiric acid	449.56 ± 1.69 ^{a}	1.16	429.01 ± 12.17 ^{a}	0.94
Valine *	1353.73 ± 33.90 ^{b}	3.49	2473.47 ± 47.92 ^{a}	5.41
β-aminobutiric acid	9.57 ± 0.05 ^{b}	0.03	10.07 ± 0.14 ^{a}	0.02
Leucine *	305.04 ± 8.16 ^{a}	0.79	352.93 ± 17.06 ^{a}	0.77
Isoleucine *	518.95 ± 21.26 ^{a}	1.34	590.55 ± 12.22 ^{a}	1.29
Threonine *	661.81 ± 0.46 ^{a}	1.71	-	-
Serine	828.72 ± 5.45 ^{b}	2.14	10,207.99 ± 97.43 ^{a}	22.33
Proline	2761.80 ± 4.36 ^{a}	7.12	1315.84 ± 29.51 ^{b}	2.88
Asparagine	2931.65 ± 26.37 ^{a}	7.56	2435.22 ± 51.38 ^{b}	5.33
Thioproline	1991.27 ± 10.83 ^{a}	5.13	1163.17 ± 21.95 ^{b}	2.55
Aspartic acid	5912.94 ± 204.58 ^{a}	15.24	1866.28 ± 46.35 ^{b}	4.08
Methionine *	1015.11 ± 0.91 ^{a}	2.62	648.43 ± 25.56 ^{b}	1.42
3/4-Hidroxiproline	672.65 ± 0.26 ^{a}	1.73	708.95 ± 41.18 ^{a}	1.5
Phenylalanine *	444.97 ± 27.45 ^{b}	1.15	735.80 ± 3.49 ^{a}	1.61
Glutamic acid	7728.82 ± 116.04 ^{a}	19.92	1930.72 ± 23.71 ^{b}	4.22
α-aminoadipic acid	462.50 ± 1.18 ^{b}	1.19	569.67 ± 2.24 ^{a}	1.25
Glutamine	7728.82 ± 116.04 ^{b}	3.21	8818.06 ± 4.69 ^{a}	19.29
Ornithine	-	-	597.93 ± 3.11 ^{a}	1.31
Glycylproline	508.17 ± 2.77 ^{a}	1.31	472.08 ± 8.71 ^{b}	1.03
Hidroxylysine	528.23 ± 3.18 ^{a}	1.36	524.77 ± 2.24 ^{a}	1.15
Proline-Hydroxyproline	508.41 ± 2.97 ^{a}	1.31	446.33 ± 3.21 ^{b}	0.98
Histidine *	779.33 ± 11.84 ^{b}	2.01	1042.54 ± 77.08 ^{a}	2.28
Lysine *	601.01 ± 2.18 ^{a}	1.55	596.36 ± 68.16 ^{a}	1.31
Tyrosine	458.58 ± 9.18 ^{a}	1.18	462.59 ± 20.18 ^{a}	1.01
Tryptophan *	532.58 ± 1.83 ^{a}	1.37	534.27 ± 0.68 ^{a}	1.17
Cystathionine	649.52 ± 7.04 ^{a}	1.67	661.12 ± 0.81 ^{a}	1.45
Cystine	662.08 ± 2.45 ^{b}	1.71	732.86 ± 1.66 ^{a}	1.60
Total AAs	38,795.21		45,710.55	
Essential AAs, %	16.01		15.26	
Non-essential AAs, %	83.99		84.74	

AAs: amino acids. The symbol * means essential AAs. Values are presented as mean ± standard deviation. When followed by different superscript letters (^{a,b}), values are statistically different at the 95% confidence level.

The meal had a significantly ($p < 95\%$) higher content of total AAs (38,795.21 nmol/g in seeds vs. 45,710.55 nmol/g in meal). The most abundant amino acid found in the seeds was glutamic acid, followed by aspartic acid, asparagine, proline, alanine and glycine. In meal, the highest AA content found was that for serine, followed by glutamine, glycine, alanine and asparagine. The lowest contents of AAs identified in the seeds and the meal were those of α-aminobutiric acid, β-aminobutiric acid, leucine and proline/hydroxyproline.

For all the AAs, relative percentages were calculated, with which the total percentage of essential/non-essential AAs was calculated. The essential AAs investigated were valine, leucine, isoleucine, threonine, methionine, phenylalanine, histidine and tryptophan. Leucine, isoleucine and valine are named branched-chain AAs. They must be obtained through our diet and cannot be synthetized by the body. These AAs are the building blocks for the synthesis of proteins that play an important role in the body's energy metabolism [1].

In the meal, the essential AAs represented 15.26%, while in the seeds, they represented 16.01%. Valine was the major AA present in both the seeds and the meal.

In seeds, the pattern of the other essential AAs was methionine > histidine > threonine > lysine > tryptophan > isoleucine > phenylalanine > leucine.

Threonine was the only essential AA that was not present in the meal. In decreasing order, the other essential AAs present in the meal were histidine > methionine > lysine > isoleucine > tryptophan > leucine. The values found are in the range found in the literature (13.13–19.26) [60–63].

3.4. Functional Properties

Functional properties are important in food product manufacturing, transportation, storage and stability [64]. The functional properties investigated were bulk density (BD), water holding capacity (WHC), oil holding capacity (OHC), emulsion capacity/stability (EC/ES), foam capacity/stability (FS/FC) and least gelatinization concentration (LGC). The values found for rapeseed meal were 0.5942 g/mL, 2.92 g/g, 1.29 g/g, 25.65%, 93.33%, 6.47% and 16%, respectively, for BD, WHC, OHC, EC, ES, FC and LGC (Table 9).

Table 9. Functional and color properties of rapeseed meal.

Parameter	BD (g/mL)	WHC (g/g)	OHC (g/g)	EC (%)	ES (%)	FC (%)	LGC (%)	L*	a*	b*
SR	0.5942	2.92	1.29	25.65	93.33	6.47	16.00	63.29	2.14	18.29
±SD	0.004	0.18	0.05	1.19	5.77	0.56	0.00	0001	0.02	0.01

SR—rapeseed meal; SD—standard deviation; BD—bulk density; WHC—water holding capacity; OHC—oil holding capacity; EC—emulsion capacity; ES—emulsion stability; FC—foaming capacity: LGC—least gelatinization concentration L*—lightness; a*—redness; b*—yellowness.

The OHC represents the amount of fat absorbed by the non-polar side of the protein chain, while the WHC represents the amount of water absorbed by the polar side of the protein chain. The interaction of water and oil with rapeseed meal is important in food formulations due to its influence on its taste and texture [65]. The fat acts as an enhancer of mouthfeel and a flavor retainer [66]. The values found in the literature for OHC and WHC were 0.90–7 g/g and 1.1–6.2 g/g, respectively [4,33,67–70].

BD is the property that describe a powder's weight and depends on the size, shape and state of compaction of the powder particles [30,71]. The results obtained were in accordance with those found in the literature (0.498–0.726 g/mL) [72–74].

EC measures the amount of protein that mixes with oil. An emulsion is a two-phase system that includes immiscible liquids (water and oil) [66]. The emulsion stability measures the amount of water released by an emulsion over time [2]. These parameters depend on proteins' flexibility and hydrophobicity, which improve the molecular anchoring of the oil/water interface and thus more stable emulsions are obtained [30]. The values obtained were in the range found in the literature (20.00–56.60% for EC and 70–108% for ES) [33,67,68,70].

LGC represent the minimum concentration of powder necessary to form a gel. The process of gelation implies the transformation of a viscous liquid into a three dimensional viscous–elastic matrix due to the swelling of proteins and starch through ordered polymerization of the molecules by heating [64]. The concentration obtained in our study was 16%. Similar values was found in the literature (14.90–15.70%) [68].

Regarding the color, the rapeseed meal powder was dark (a low lightness value) with a reddish and yellowish tone (values for a* and b* were positive).

The FC is a measure of the interfacial area formed by a protein during foaming [75]. The rapeseed meal showed a low foaming (6.47%) capacity, but the foam had high stability. The foam decreased from 100% to 68.18% within an hour (Figure 2).

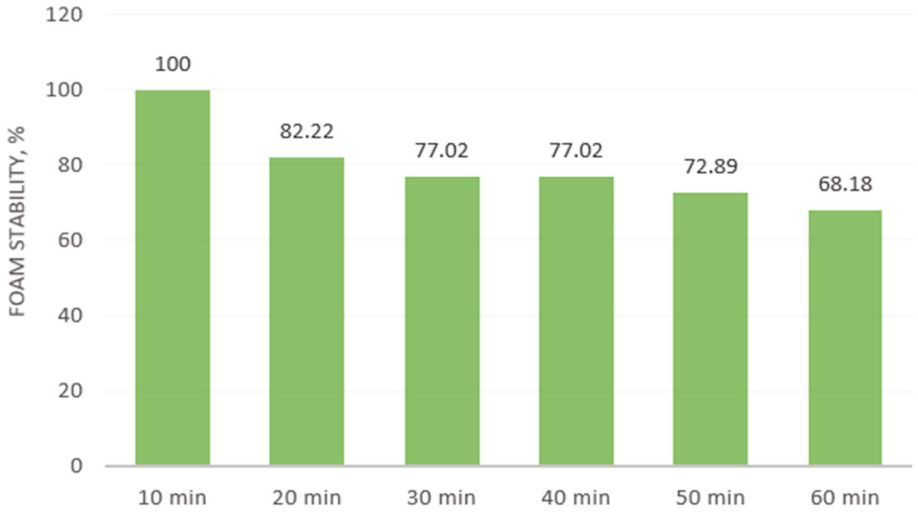


Figure 2. Foaming stability of rapeseed meal within 1 h.

The powder wettability (depending on time and the dispersion behavior) was recorded as good. On contact with water, the meal powder gradually moistens. Part of the powder is dispersed in the liquid, and the other is deposited at the bottom of the Berzelius beaker. After a few seconds, everything fades to the background. After half an hour, all of the powder particles settle at the bottom of the Berzelius beaker. By vortexing, the sample is dispersed through the liquid.

3.5. Qualitative Analysis of Main Functional Groups

Fourier Transform Infrared Attenuated Total Reflectance (FTIR-ATR) can be considered a green, rapid and innovative method for characterizing samples. This method furnishes the chemical and biochemical characteristic substances in a sample by recording their molecular vibration (torsion, bending and stretching of the chemical bonds).

The FT-IR spectra presented nine wavenumbers (Figure 3) in three different spectra zones, namely 4000–2500 cm^{-1} (the single-bond region), 2000–1500 cm^{-1} (the double-bond region) and 1500–600 cm^{-1} (the fingerprint region).

In the first region, there were three absorption wavenumbers, namely 3283.24 cm^{-1}, 2922.41 cm^{-1} and 2852.96 cm^{-1}, possibly attributed to the OH stretching modes of polysaccharides and/or cellulose [76] and asymmetric and symmetric stretching vibrations, mainly associated with the hydrocarbon chain found in fatty acids [77].

In the second region, the spectral band at 1743.98 cm^{-1} can possibly be attributed to the C=O bonds of the ester group. This is related to the presence of fatty acids and their carbohydrates, pectins and lignin [78]. The peaks at 1640.58 cm^{-1} and 1540.88 cm^{-1} were amidic bands I and II, respectively [79]; these result from the C=O stretching of amides (which involves the carbonyl stretching vibration of the peptide backbone) and bending variations in the N-H groups [56].

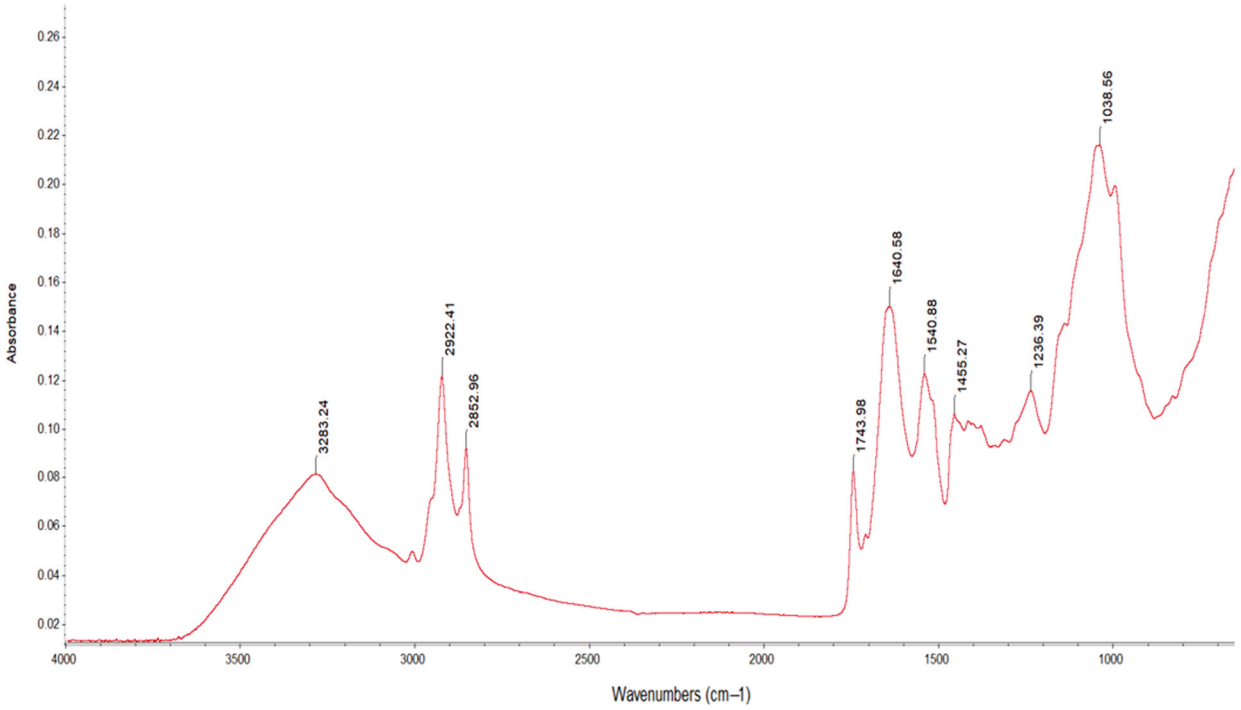


Figure 3. FTIR spectra for rapeseed meal.

The fingerprint region was rich in peaks (from various stretching, bending, rocking, scissoring and torsional modes), providing information about the organic compounds. Unfortunately, due to its complexity, it is difficult to analyze [80]. The spectral band at 1455.27 cm^{-1} possibly corresponded to aromatic C-C stretching related to the presence of phenolic compounds, while the bending vibration of OH at 1236.39 cm^{-1} and the C-O stretching at 1038.56 cm^{-1} may have been related to polysaccharide substances [81,82].

3.6. Model Fitting and Statistical Analysis

The rapeseed meal extracts were obtained according to the information presented in Table 10. A total of 45 experiments were conducted with various combinations of independent variables (A, temperature, B, time, C, amplitude used for ultrasound-assisted treatment and D, solvent used for extraction). The experimental data recorded for the total polyphenol content and 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity are also presented in Table 10.

The ANOVA for the model fitting showed that the mathematic model chosen was statistically significant ($p < 0.0001$) and predicted the responses accurately. Total polyphenol content and antioxidant activity according to the DPPH data were fitted to a quadratic model, which explained 94% and 91% of the data variation, respectively.

For the experimental data, a multiple regression analysis was applied, obtaining a quadratic polynomial, in Equation (16), where R represents the responses (TPC and DPPH), x_0 is the constant and b_1-b_{16} are the coefficients of regression found in Table 11:

$$\begin{gathered} R = b_0 + b_1 \times A + b_2 \times B + b_3 \times C + b_4 \times D1 + b_5 \times D2 + b_6 \times AB + b_7 \times AC \\ + b_8 \times AD1 + b_9 \times AD2 + b_{10} \times BC + b_{11} \times BD1 + b_{12} \times BD2 + b_{13} \times CD1 + b_{14} \times CD2 \\ + b_{15} \times A^2 + b_{16} \times B^2 + b_{17} \times C^2 \end{gathered} \quad (16)$$

The TPC ranged from 7.14 to 193.63 mg GAE/g (the maximum values were obtained using methanol at 40 °C, for 20 min and at a 40% amplitude), and the free radical scavenging activity ranged from 40.24% to 89.07% (the maximum values were determined using methanol at 40 °C, for 10 min and at a 40% amplitude).

Table 10. Experimental design and data values.

Run	Factors								Responses	
	Coded				Actual					
	A	B	C	D	Temperature, °C	Time, min	Amplitude, %	Solvent	TPC, mg GAE/g	DPPH, %
1	−1	−1	0	{ 1 0 }	30	10	70	water	53.41	71.50
2	1	−1	0	{ 1 0 }	50	10	70	water	111.84	77.61
3	−1	1	0	{ 1 0 }	30	20	70	water	126.88	83.00
4	1	1	0	{ 1 0 }	50	20	70	water	102.05	78.47
5	−1	0	−1	{ 1 0 }	30	15	40	water	30.00	65.55
6	1	0	−1	{ 1 0 }	50	15	40	water	46.00	80.19
7	−1	0	1	{ 1 0 }	30	15	100	water	142.32	81.70
8	1	0	1	{ 1 0 }	50	15	100	water	117.00	56.93
9	0	−1	−1	{ 1 0 }	40	10	40	water	19.00	78.47
10	0	1	−1	{ 1 0 }	40	20	40	water	104.48	75.02
11	0	−1	1	{ 1 0 }	40	10	100	water	174.00	75.00
12	0	1	1	{ 1 0 }	40	20	100	water	151.4	74.16
13	0	0	0	{ 1 0 }	40	15	70	water	47.00	83.63
14	0	0	0	{ 1 0 }	40	15	70	water	46.92	76.31
15	0	0	0	{ 1 0 }	40	15	70	water	46.92	76.14
16	−1	−1	0	{ 0 1 }	30	10	70	ethanol	47.31	44.53
17	1	−1	0	{ 0 1 }	50	10	70	ethanol	75.15	66.33
18	−1	1	0	{ 0 1 }	30	20	70	ethanol	63.35	72.22
19	1	1	0	{ 0 1 }	50	20	70	ethanol	25.00	58.00
20	−1	0	−1	{ 0 1 }	30	15	40	ethanol	7.14	40.24
21	1	0	−1	{ 0 1 }	50	15	40	ethanol	45.84	65.49
22	−1	0	1	{ 0 1 }	30	15	100	ethanol	51.54	67.00
23	1	0	1	{ 0 1 }	50	15	100	ethanol	9.41	41.08
24	0	−1	−1	{ 0 1 }	40	10	40	ethanol	35.00	50.00
25	0	1	−1	{ 0 1 }	40	20	40	ethanol	35.02	63.80
26	0	−1	1	{ 0 1 }	40	10	100	ethanol	84.29	54.00
27	0	1	1	{ 0 1 }	40	20	100	ethanol	47.77	70.00
28	0	0	0	{ 0 1 }	40	15	70	ethanol	8.00	80.00
29	0	0	0	{ 0 1 }	40	15	70	ethanol	8.00	70.00
30	0	0	0	{ 0 1 }	40	15	70	ethanol	8.00	70.00
31	−1	−1	0	{ −1 −1 }	30	10	70	methanol	112.43	82.34
32	1	−1	0	{ −1 −1 }	50	10	70	methanol	83.00	81.00
33	−1	1	0	{ −1 −1 }	30	20	70	methanol	175.72	74.00
34	1	1	0	{ −1 −1 }	50	20	70	methanol	81.06	73.09
35	−1	0	−1	{ −1 −1 }	30	15	40	methanol	83.00	70.56
36	1	0	−1	{ −1 −1 }	50	15	40	methanol	128.39	87.40
37	−1	0	1	{ −1 −1 }	30	15	100	methanol	141.21	78.55
38	1	0	1	{ −1 −1 }	50	15	100	methanol	28.14	49.54
39	0	−1	−1	{ −1 −1 }	40	10	40	methanol	118.3	89.07
40	0	1	−1	{ −1 −1 }	40	20	40	methanol	193.63	82.34
41	0	−1	1	{ −1 −1 }	40	10	100	methanol	135.89	81.50
42	0	1	1	{ −1 −1 }	40	20	100	methanol	114.21	70.00
43	0	0	0	{ −1 −1 }	40	15	70	methanol	67.00	86.00
44	0	0	0	{ −1 −1 }	40	15	70	methanol	68.00	85.70
45	0	0	0	{ −1 −1 }	40	15	70	methanol	66.79	86.00

The values found in the literature were 6.97–21.04 mg GAE/g for total polyphenol content and 6.59–69.08% for antioxidant activity [4,77,83,84]. The TPC and the antioxidant activity are dependent on the type of solvent used, its concentration, the granularity of the sawdust and the temperature. Teh et al. [83] investigated the effects of using different solvents with varying concentrations on the polyphenol content, obtaining high values using a mixture of solvents (methanol:acetone:water), followed by 80% acetone, 80% methanol and ethanol. Nandasiri et al. [84], obtaining high values for antioxidant activity,

used 70% methanol and ethanol and high temperatures. The meal fraction particles (below 250 μm) led to the highest values for these two responses [4].

Table 11. ANOVA results for model fitting (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). Coefficients of regression that explain the effect of the variables and their interactions.

Factors	TPC	DPPH
R^2	0.9469	0.9111
Adjusted R^2	0.9134	0.8551
F value	28.30	16.27
p value	<0.0001	<0.0001
Lack of Fit	0.2857	0.3560
b_0	+44.74	+79.31
b_1	−7.56 *	−0.67
b_2	+7.12 *	+0.95
b_3	+14.64 ***	−2.03 *
b_4	+12.51 ***	+3.95 ***
b_5	−41.12 ***	−10.79 ***
b_6	−17.89 ***	−3.85 **
b_7	−23.38 ***	−11.37 ***
b_8	+10.59 **	−0.40
b_9	+5.82 **	+1.53
b_{10}	−20.14 ***	+0.003
b_{11}	+8.70 **	+0.061 ***
b_{12}	−15.95 **	+5.20 ***
b_{13}	+33.51 ***	+0.60 **
b_{14}	−5.89 ***	+3.60 **
b_{15}	+5.72	−7.03 ***
b_{16}	+37.64 ***	−0.44
b_{17}	+18.71 **	−6.93 ***

Phenolic acids and flavonoids are the main contributors to antioxidant activity. This correlation was demonstrated by Sepahpour et al. [85] for four different plants (turmeric, curry leaf, ginger and lemongrass), and generally, a high TPC was correlated with high DPPH values.

3.7. Total Polyphenolic Content

Temperature (A) had a significant negative effect on polyphenol content ($p < 0.05$), while time and amplitude (B and C) had a significant positive effect ($p < 0.05$ and $p < 0.001$, respectively). The highest positive effect was observed for the quadratic term of time (B^2). From the equation, it can be observed that the interaction between temperature and time had the highest negative effect on TPC.

The variables temperature and amplitude had a greater positive effect on TPC when water was used as the extraction solvent. On the other hand, the variable time had a greater effect when the solvent used was ethanol. The combination of all variables had a negative effect on TPC for all the solvents used, and the strongest negative effect was observed for the combination of time and solvent (in this case ethanol), followed by that of temperature and time.

A 3D picture that shows the effects of different treatments on the TPC is presented in Figure 4.

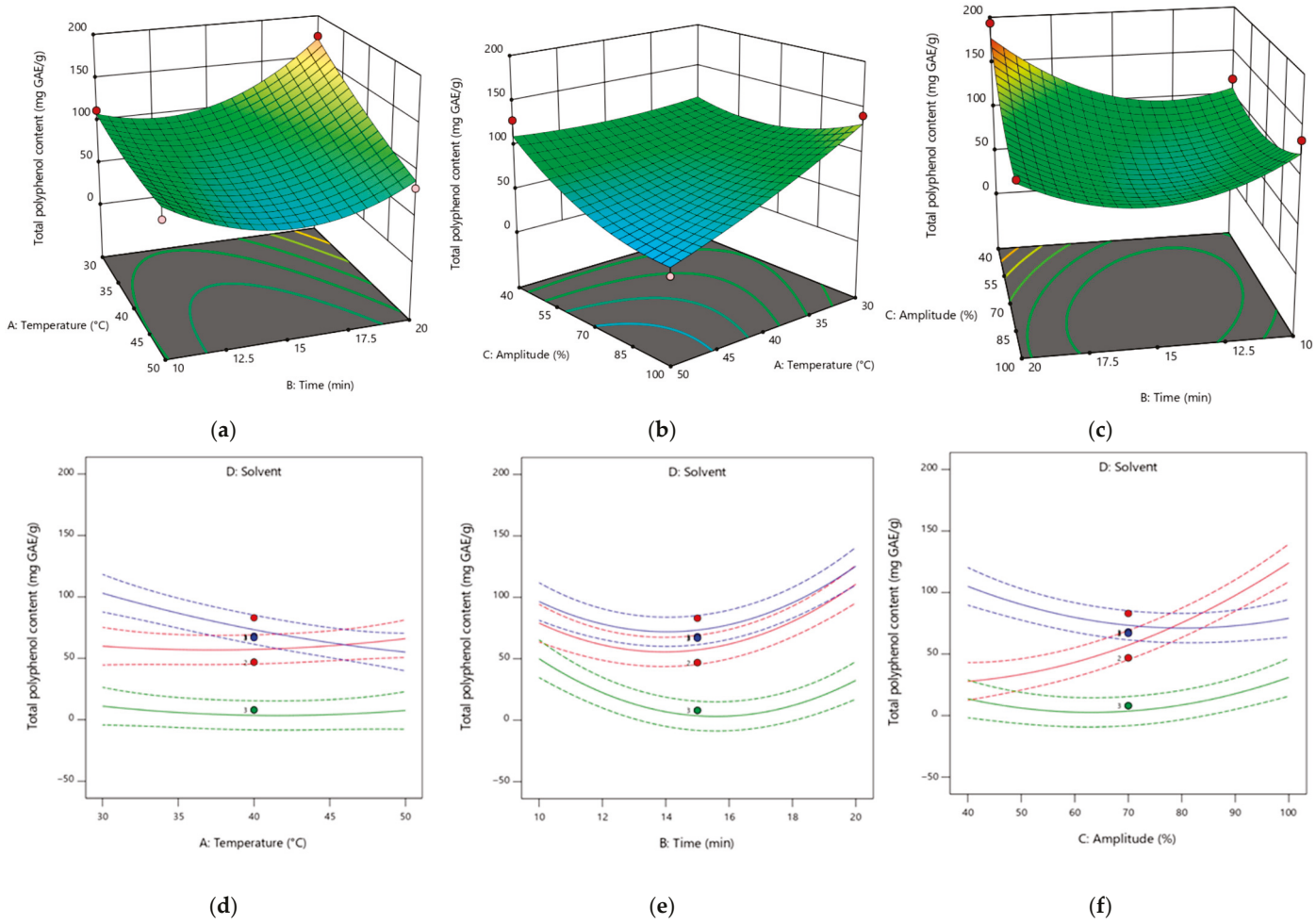


Figure 4. Response surface plots showing the effect of the extraction parameters and different solvents on the phenolic compounds. Pictures (**a**–**c**) are for the extraction in methanol. For pictures (**d**–**f**), the color green corresponds to ethanol, blue corresponds to methanol and red corresponds to water.

3.8. DPPH Free Radical Scavenging Activity

Amplitude (C) had a significant ($p < 0.05$) negative effect on antioxidant activity, while temperature and time (A and B) had no significant effect. The largest positive effect was observed for the interaction between time and solvent2 (BD2-ethanol). On the other hand, solvent D2 (ethanol) had the greatest negative effect on antioxidant capacity.

Temperature had a greater positive effect on antioxidant activity when water was used as the extraction solvent. On the other hand, time and amplitude had a greater effect when the solvent used was ethanol. Only the combination of time and amplitude had a positive effect on antioxidant activity for all solvents used.

The surface responses in Figure 5 show the effect of different factors on the evolution of antioxidant activity.

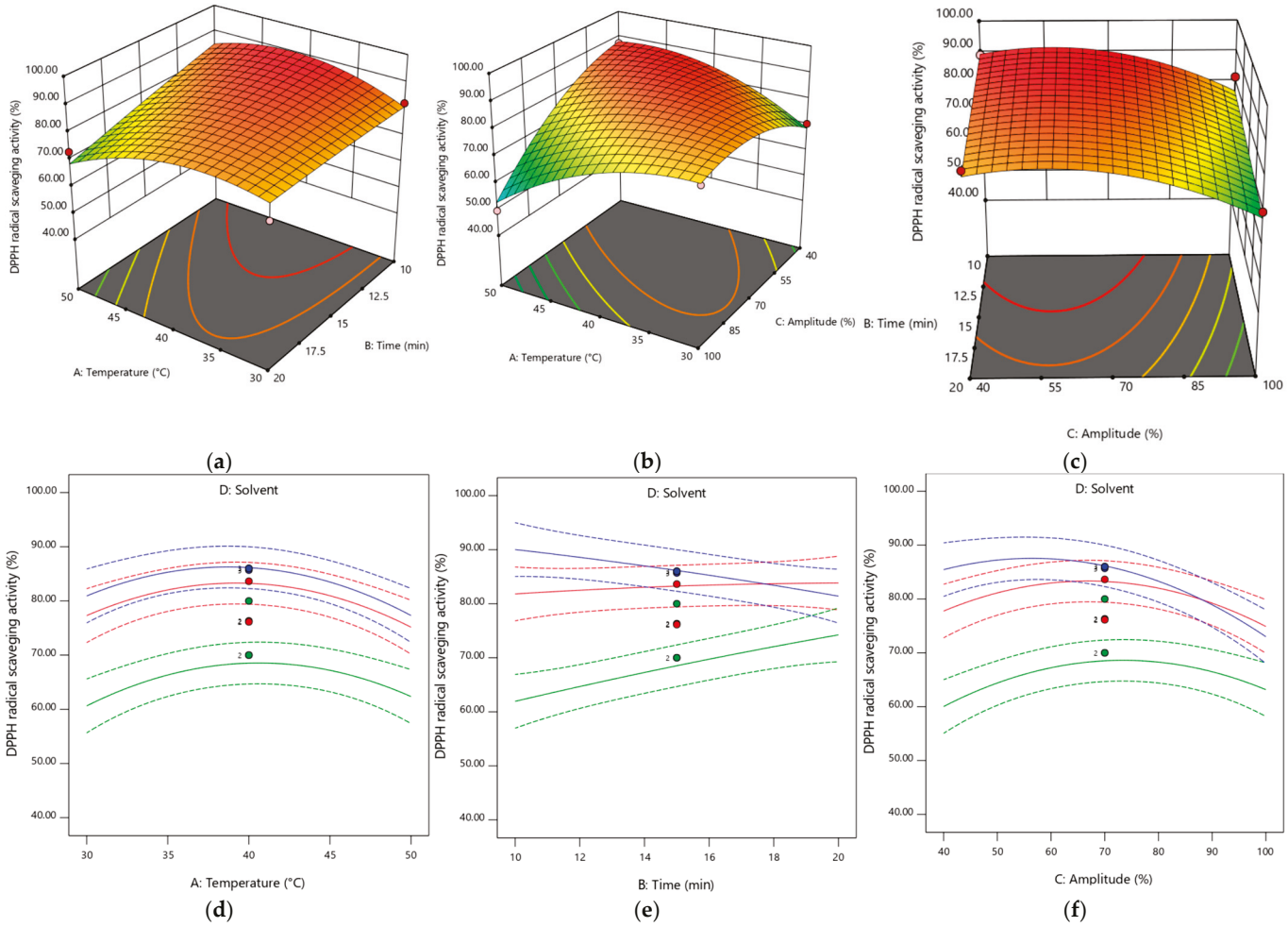


Figure 5. Response surface plots showing the effect of the extraction parameters and different solvents on the antioxidant activity. Pictures (**a**–**c**) are for the extraction in methanol. For pictures (**d**–**f**), the color green corresponds ethanol, blue corresponds to methanol and red corresponds to water.

3.9. HPLC Analysis of Phenolic Compounds

The phenolic acids were determined for the extract with the highest TPC. The average concentrations found of the phenolic acids in the rapeseed meal are presented in Table 12.

Table 12. Phenolic compounds in rapeseed meal.

Phenolic Acids	Results, mg/kg
4-Hydroxybenzoic acid	155.49 ± 5.80
Vanillic acid	2496.71 ± 46.88
Caffeic acid	1885.43 ± 54.27
Chlorogenic acid	1891.18 ± 19.53
p-cumaric acid	30.53 ± 0.92
Rosmarinic acid	354.09 ± 11.29
Myricetin	1966.51 ± 179.52
Luteolin	17.89 ± 0.64
Quercetin	460.45 ± 30.72
Kaempferol	14.50 ± 0.92
Total	9272.28

The most abundant phenolic compound was vanillic acid, followed by myricetin, chlorogenic acid and caffeic acid. The concentration sum for the phenolic compounds

detected accounted for 9272.28 mg per kg of rapeseed meal. This value is in the range found in the literature (410–21,602.9 mg/kg) [56,83,86]. The total obtained after summing the individual phenols was lower compared to the overall total obtained using the Folin–Ciocâlteu method because this reagent interacts not only with phenols but also with UFAs, AAs, carbohydrates, proteins and vitamins [43].

There are several studies in the literature regarding the phenolic components of rapeseed meal. Teh et al. [83] found gallic acid, p-coumaric, catechin, caffeic acid, epicatechin, ferulic acid, quercetin and luteolin. Meanwhile, Di Lena et al. [56] in addition found protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, chlorogenic acid, neochlorogenic acid, syringic acid, cryptochlorogenic acid, sinapic acid and cinnamic acid.

4. Conclusions

The increased popularity of the sustainability concept has led industrial production to develop new strategies for the efficient use of all resources without creating waste. Rapeseeds are grains used primarily for oil production. The extraction process generates large amounts of rapeseed meal, which is currently under-used.

The results of this study highlight the nutritional features of rapeseeds and meal after oil extraction. Cold-pressed rapeseed meal showed great potential for the formulation of various foodstuffs due to its high protein, mineral and dietary fiber contents.

The meal is considered safe to use because its mycotoxin content was in the allowed range and heavy metals were absent. Furthermore, its water activity index was low < 0.4, which means it does not allow for microorganism development.

Mainly, rapeseed meal is a valuable source of protein (35.04%), which includes essential amino acids (15.26%), especially valine. Rapeseed meal is rich in minerals such as Mg, Ca Se, Ce, Tl, Mn, Cr, Zn, Ti, Be, Fe II, Fe III, Co, Ni and Mo. Regarding its dietary fiber content, rapeseed meal can meet the consumer demand for fiber-rich food.

The meal also presented good water-holding and fat absorption capacities, emulsifying and foaming activities and stability. These important factors make rapeseed meal suitable for use in products in which hydration and viscosity improvements are necessary.

The total polyphenolic content and antioxidant activity values were affected by both the ultrasonic treatment and the solvent used. The best values were obtained when methanol was used for the extraction. Of all the parameters applied, the temperature applied had the most negative influence on TPC. The amplitude and time of ultrasonication positively influences both the TPC and the antioxidant effect of the rapeseed meal. Based on the mathematical models obtained, the extraction process can be optimized to obtain the maximum yield in terms of the total content of polyphenols with a high antioxidant activity.

Rapeseed meal is a low-cost, renewable resource rich in bioactive compounds. Due to its rich content in desirable nutrients, its use in the food industry is inevitable, not only for nutritional purposes but also due to its beneficial impact in terms of its textural and antioxidant qualities. It can be used as a co-product in the food industry for the manufacture of novel high-value-added products or supplements (through the extraction of proteins and dietary fibers). Other future directions for this valuable by-product lie in the realization of edible and biodegradable food packaging materials.

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References

1. Melo, D.; Álvarez-Ortí, M.; Nunes, M.A.; Costa, A.S.G.; Machado, S.; Alves, R.C.; Pardo, J.E.; Oliveira, M.B.P.P. Whole or defatted sesame seeds (*Sesamum indicum* L.)? The effect of cold pressing on oil and cake quality. *Foods* **2021**, *10*, 2108. [CrossRef] [PubMed]
2. Petraru, A.; Ursachi, F.; Amariei, S. Nutritional characteristics assessment of sunflower seeds, oil and cake. Perspective of using sunflower oilcakes as a functional ingredient. *Plants* **2021**, *10*, 2487. [CrossRef] [PubMed]
3. Borrello, M.; Caracciolo, F.; Lombardi, A.; Pascucci, S.; Cembalo, L. Consumers' perspective on circular economy strategy for reducing food waste. *Sustainability* **2017**, *9*, 141. [CrossRef]
4. Bárta, J.; Bártová, V.; Jarošová, M.; Švajner, J.; Smetana, P.; Kadlec, J.; Filip, V.; Kyselka, J.; Berčíková, M.; Zdráhal, Z.; et al. Oilseed Cake Flour Composition, Functional Properties and Antioxidant Potential as Effects of Sieving and Species Differences. *Foods* **2021**, *10*, 2766. [CrossRef]
5. Zhu, H.; Wang, L.; Li, X.; Shi, J.; Scanlon, M.; Xue, S.; Nosworthy, M.; Vafaei, N. Canola Seed Protein: Pretreatment, Extraction, Structure, Physicochemical and Functional Characteristics. *Foods* **2024**, *13*, 1357. [CrossRef]
6. Wanasundara, J.P.D.; Tan, S.; Alashi, A.M.; Pudel, F.; Blanchard, C. *Proteins from Canola/Rapeseed: Current Status*; Elsevier: Amsterdam, The Netherlands, 2017; ISBN 9780128027769.
7. Lomascolo, A.; Uzan-Boukhris, E.; Sigoillot, J.C.; Fine, F. Rapeseed and sunflower meal: A review on biotechnology status and challenges. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 1105–1114. [CrossRef]
8. Petraru, A.; Amariei, S. Oil press-cakes and meals valorization through circular economy approaches: A review. *Appl. Sci.* **2020**, *10*, 7432. [CrossRef]
9. Kumar, S.; Kushwaha, R.; Verma, M.L. *Recovery and Utilization of Bioactives from Food Processing Waste*; Elsevier: Amsterdam, The Netherlands, 2019; ISBN 9780444643230.
10. Gupta, A.; Sharma, R.; Sharma, S.; Singh, B. Oilseed as Potential Food Ingredient. In *Trends & Prospects in Food Technology, Processing and Preservation*, 1st ed.; Prodyut Kumar, P., Mahawar, M.K., Abobatta, W., Panja, P., Eds.; Today and Tomorrow's Printers and Publishers: New Delhi, India, 2018; pp. 25–58.
11. Tekeba Nega, Y. woldes Review on Nutritional Limitations and Opportunities of using Rapeseed Meal and other Rape Seed by—Products in Animal Feeding. *J. Nutr. Health Food Eng.* **2018**, *8*, 43–48. [CrossRef]
12. Otles, S.; Despoudi, S.; Bucatariu, C.; Kartal, C. Food waste mangement, valorization, and sustainability in the food industry. In *Food Waste Recovery*; Galanakis, C.M., Ed.; Elsevier: London, UK, 2015; pp. 3–23. ISBN 978-0-12-800351-0.
13. Mateos-Aparicio, I.; Matias, A. *Food Industry Processing By-Products in Foods*; Galanakis, C.M., Ed.; Academic Press: London, UK, 2019; ISBN 9780128164532.
14. Abedini, A.; Alizadeh, A.M.; Mahdavi, A.; Golzan, S.A.; Salimi, M.; Tajdar-Oranj, B.; Hosseini, H. Oilseed Cakes in the Food Industry: A Review on Applications, Challenges, and Future Perspectives. *Curr. Nutr. Food Sci.* **2021**, *17*, 345–362. [CrossRef]
15. Popović, S.; Hromiš, N.; Šuput, D.; Bulut, S.; Romanić, R.; Lazić, V. *Valorization of By-Products from the Production of Pressed Edible Oils to Produce Biopolymer Films*, 1st ed.; Academic Press: London, UK, 2020; ISBN 9780128181881.
16. Zago, E.; Lecomte, J.; Barouh, N.; Aouf, C.; Carré, P.; Fine, F.; Villeneuve, P. Influence of rapeseed meal treatments on its total phenolic content and composition in sinapine, sinapic acid and canolol. *Ind. Crops Prod.* **2015**, *76*, 1061–1070. [CrossRef]
17. Dranca, F.; Oroian, M. Ultrasound-Assisted Extraction of Pectin from Malus domestica 'Fălticeni' Apple Pomace. *Processes* **2019**, *7*, 488. [CrossRef]
18. Mihalcea, A.; Amariei, S. Study on Contamination with Some Mycotoxins in Maize and Maize-Derived Foods. *Appl. Sci.* **2022**, *12*, 2579. [CrossRef]
19. *ISO 665:2020*; Animal and Vegetable Fats and Oils—Determination of Moisture and Volatile Matter Content. International Organization for Standardization: Geneva, Switzerland, 2020.
20. AOAC. Moisture in malt. Method 935.29. In *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 2003.
21. AOAC. Protein (crude) in nuts and nut products. Method 950.48. In *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 2003.
22. AOAC. Ash of Flour (Direct Method), Method 923.03. In *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 2003.
23. *ISO 659:2020*; Animal and Vegetable Fats and Oils—Determination of Oil Content. International Organization for Standardization: Geneva, Switzerland, 2020.
24. AOAC. Fat (Crude) or Ether Extract in Animal Feed, Method 985.29. In *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 2003.
25. AOAC. Total dietary fiber in foods, enzymatic-Gravimetric, Method 985.29. In *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 2003.
26. Konak, M.; Çarman, K.; Aydin, C. Physical properties of chick pea seeds. *Biosyst. Eng.* **2002**, *82*, 73–78. [CrossRef]
27. Omowaye-Taiwo, O.A.; Fagbemi, T.N.; Ogunbusola, E.M.; Badejo, A.A. Effect of germination and fermentation on the proximate composition and functional properties of full-fat and defatted cucumeropsis mannii seed flours. *J. Food Sci. Technol.* **2015**, *52*, 5257–5263. [CrossRef] [PubMed]
28. Bhise, S.R.; Kaur, A.; Manikantan, M.R.; Singh, B. Development of textured defatted sunflower meal by extrusion using response surface methodology. *Acta Aliment.* **2015**, *44*, 251–258. [CrossRef]

29. Marasinghe, S.S.K.; Yalegama, C.; Pathirana, D.T.H.; Marikkar, J.M.N. The Physical and Functional Properties of Partially Defatted Coconut Testa Flour. *Int. J. Ofcoconut Res. Dev.* **2021**, *37*, 11–22.
30. Rani, R.; Badwaik, L.S. Functional Properties of Oilseed Cakes and Defatted Meals of Mustard, Soybean and Flaxseed. *Waste Biomass Valorization* **2021**, *12*, 5639–5647. [CrossRef]
31. North, K.; North, K. Nutritional and functional characterization of defatted seed cake flour of two Sudanese groundnut (*Arachis hypogaea*) Cultivars. *Int. Food Res. J.* **2012**, *19*, 629–637.
32. Iyenagbe, D.O.; Malomo, S.A.; Idowu, A.O.; Badejo, A.A.; Fagbemi, T.N. Effects of thermal processing on the nutritional and functional properties of defatted conophor nut (*Tetracarpidium conophorum*) flour and protein isolates. *Food Sci. Nutr.* **2017**, *5*, 1170–1178. [CrossRef]
33. Naczk, M.; Diosady, L.L.; Rubin, L.J. Functional Properties of Canola Meals Produced by a Two-phase Solvent Extraction System. *J. Food Sci.* **1985**, *50*, 1685–1688. [CrossRef]
34. Petrović, M.; Kezić, N.; Bolanča, V. Optimization of the GC method for routine analysis of the fatty acid profile in several food samples. *Food Chem.* **2010**, *122*, 285–291. [CrossRef]
35. Senila, L.; Neag, E.; Cadar, O.; Kovacs, M.H.; Becze, A. Chemical, Nutritional and Antioxidant Characteristics of Different Food Seeds. *Appl. Sci.* **2020**, *10*, 1589. [CrossRef]
36. Dabadé, D.S.; Jacxsens, L.; Miclotte, L.; Abatih, E.; Devlieghere, F.; De Meulenaer, B. Survey of multiple biogenic amines and correlation to microbiological quality and free amino acids in foods. *Food Control.* **2021**, *120*, 107497. [CrossRef]
37. Gifty, A.G.; De Meulenaer, B.; Olango, T.M. Variation in tuber proximate composition, sugars, fatty acids and amino acids of eight Oromo dinich (*Plectranthus edulis*) landraces experimentally grown in Ethiopia. *J. Food Compos. Anal.* **2018**, *67*, 191–200. [CrossRef]
38. Petraru, A.; Amariei, S. Sunflower Oilcake as a Potential Source for the Development of Edible Membranes. *Membranes* **2022**, *12*, 789. [CrossRef]
39. Taha, F.; Mohamed, G.A.; Mohamed, S.H.; Mohamed, S.S. Optimization of the Extraction of Total Phenolic Compounds from Sunflower Meal and Evaluation of the Bioactivities of Chosen Extracts. *Am. J. Food Technol.* **2011**, *6*, 1002–1020. [CrossRef]
40. Zardo, I.; de Espíndola Sobczyk, A.; Marczak, L.D.F.; Sarkis, J. Optimization of Ultrasound Assisted Extraction of Phenolic Compounds from Sunflower Seed Cake Using Response Surface Methodology. *Waste Biomass Valorization* **2019**, *10*, 33–44. [CrossRef]
41. Soufi, O.; Medouni-Haroune, L.; Bachirbey, M.; Medouni-Adrar, S.; Idir, F.; Heddad, T.; Ouldsaadi, L.; Romero, C.; Madani, K.; Makhlouf-Boulekbache, L. Statistical optimization of ultrasound-assisted extraction of polyphenols from olive pomace. *Sustain. Chem. Pharm.* **2023**, *36*, 101260. [CrossRef]
42. Lavilla, I.; Bendicho, C. *Fundamentals of Ultrasound-Assisted Extraction*; Elsevier: Amsterdam, The Netherlands, 2017; ISBN 9780128096154.
43. Chetrariu, A.; Dabija, A. Spent Grain from Malt Whisky: Assessment of the Phenolic Compounds. *Molecules* **2021**, *26*, 3236. [CrossRef]
44. Kenari, R.E.; Mohsenzadeh, F.; Amiri, Z.R. Antioxidant activity and total phenolic compounds of dezful sesame cake extracts obtained by classical and ultrasound-assisted extraction methods. *Food Sci. Nutr.* **2014**, *2*, 426–435. [CrossRef]
45. Izli, N.; Unal, H.; Sincik, M. Physical and mechanical properties of rapeseed at different moisture content. *Int. Agrophysics* **2009**, *23*, 137–145.
46. Baran, M.F.; Durgut, M.R.; Aktas, T.; Ulger, P.; Kayisoglu, B. Determination of Some Physical Properties of Rapeseed. *Int. J. Eng. Technol.* **2016**, *2*, 49. [CrossRef]
47. Özlü, R.; Güner, M. Determination of Pneumatic Conveying Characteristics of Canola. *J. Agric. Sci. Tarım Bilim. Derg.* **2022**, *28*, 656–665. [CrossRef]
48. Çalışır, S.; Marakoğlu, T.; Öğüt, H.; Öztürk, Ö. Physical properties of rapeseed (*Brassica napus oleifera* L.). *J. Food Eng.* **2005**, *69*, 61–66. [CrossRef]
49. Tańska, M.; Rotkiewicz, D.; Kozirok, W.; Konopka, I. Measurement of the geometrical features and surface color of rapeseeds using digital image analysis. *Food Res. Int.* **2005**, *38*, 741–750. [CrossRef]
50. Unal, H.; Sincik, M.; Izli, N. Comparison of some engineering properties of rapeseed cultivars. *Ind. Crops Prod.* **2009**, *30*, 131–136. [CrossRef]
51. Ropelewska, E.; Zapotoczny, P.; Budzyński, W.S.; Jankowski, K.J. Discriminating power of selected physical properties of seeds of various rapeseed (*Brassica napus* L.) cultivars. *J. Cereal Sci.* **2017**, *73*, 62–67. [CrossRef]
52. Razavi, S.M.A.; Yeganehzad, S.; Sadeghi, A. Moisture Dependent Physical Properties of Canola Seeds. *J. Agric. Sci. Technol.* **2007**, *11*, 309–322.
53. Gagour, J.; Ahmed, M.N.; Bouzid, H.A.; Oubannin, S.; Bijla, L.; Ibourki, M.; Hajib, A.; Koubachi, J.; Harhar, H.; Gharby, S. Proximate Composition, Physicochemical, and Lipids Profiling and Elemental Profiling of Rapeseed (*Brassica napus* L.) and Sunflower (*Helianthus annuus* L.) Grown in Morocco. *Evid. Based Complement. Altern. Med.* **2022**, *2022*, 1–12. [CrossRef]
54. Thomas, V.M.; Katz, R.J.; Auld, D.A.; Petersen, C.F.; Sauter, E.A.; Steele, E.E. Nutritional Value of Expeller Extracted Rape and Safflower Oilseed Meals for Poultry. *Poult. Sci.* **1983**, *62*, 882–886. [CrossRef]
55. Wickramasuriya, S.S.; Yi, Y.-J.; Yoo, J.; Kang, N.K.; Heo, J.M. A review of canola meal as an alternative feed ingredient for ducks. *J. Anim. Sci. Technol.* **2015**, *57*, 29. [CrossRef] [PubMed]

56. Di Lena, G.; Del Pulgar, J.S.; Lucarini, M.; Durazzo, A.; Ondrejíčková, P.; Oancea, F.; Frincu, R.M.; Aguzzi, A.; Nicoli, S.F.; Casini, I.; et al. Valorization potentials of rapeseed meal in a biorefinery perspective: Focus on nutritional and bioactive components. *Molecules* **2021**, *26*, 6787. [CrossRef] [PubMed]
57. Beyzi, E.; Gunes, A.; Buyukkilic Beyzi, S.; Konca, Y. Changes in fatty acid and mineral composition of rapeseed (*Brassica napus* ssp. *oleifera* L.) oil with seed sizes. *Ind. Crops Prod.* **2019**, *129*, 10–14. [CrossRef]
58. Gonzalez-Becerra, K.; Barron-Cabrera, E.; Muñoz-Valle, J.F.; Torres-Castillo, N.; Rivera-Valdes, J.J.; Rodriguez-Echevarria, R.; Martinez-Lopez, E. A Balanced Dietary Ratio of n-6:n-3 Polyunsaturated Fatty Acids Exerts an Effect on Total Fatty Acid Profile in RBCs and Inflammatory Markers in Subjects with Obesity. *Healthcare* **2023**, *11*, 2333. [CrossRef]
59. Yang, L.G.; Song, Z.X.; Yin, H.; Wang, Y.Y.; Shu, G.F.; Lu, H.X.; Wang, S.K.; Sun, G.J. Low n-6/n-3 PUFA Ratio Improves Lipid Metabolism, Inflammation, Oxidative Stress and Endothelial Function in Rats Using Plant Oils as n-3 Fatty Acid Source. *Lipids* **2016**, *51*, 49–59. [CrossRef]
60. Mejicanos, G.; Sanjayan, N.; Kim, I.H.; Nyachoti, C.M. Recent advances in canola meal utilization in swine nutrition. *J. Anim. Sci. Technol.* **2016**, *58*, 7. [CrossRef]
61. Zhu, X.; Chen, Y.; Hao, S.; Jin, S.; Li, X. Improvement of the Nutritional Quality of Rapeseed Meal through Solid-State Fermentation with *B. subtilis*, *S. cerevisiae*, and *B. amyloliquefaciens*. *Fermentation* **2023**, *9*, 492. [CrossRef]
62. Tuori, M. Rapeseed meal as a supplementary protein for dairy cows on grass silage-based diet, with the emphasis on the Nordic AAT-PBV feed protein evaluation system. *Agric. Food Sci.* **1992**, *1*, 367–439. [CrossRef]
63. Chen, X.; Parr, C.; Utterback, P.; Parsons, C.M. Nutritional evaluation of canola meals produced from new varieties of canola seeds for poultry. *Poult. Sci.* **2015**, *94*, 984–991. [CrossRef]
64. Nawaz, H.; Shad, M.A.; Mehmood, R.; Rehman, T.; Munir, H. Comparative Evaluation of Functional Properties of Some Commonly Used Cereal and Legume Flours and Their Blends. *Int. J. Food Allied Sci.* **2015**, *1*, 67. [CrossRef]
65. Čakarević, J.; Vidović, S.; Vladić, J.; Gavarić, A.; Jokić, S.; Pavlović, N.; Blažić, M.; Popović, L. Production of Bio-Functional Protein through Revalorization of Apricot Kernel Cake. *Foods* **2019**, *8*, 318. [CrossRef] [PubMed]
66. Hasmadi, M.; Noorfarahzilah, M.; Noraidah, H.; Zainol, M.K.; Jahurul, M.H. Functional properties of composite flour: A review. *Food Res.* **2020**, *4*, 1820–1831. [CrossRef]
67. Sosulski, F.; Humbert, E.S.; Bui, K.; Jones, J.D. Functional properties of rapeseed flours, concentrates and isolate. *J. Food Sci.* **1976**, *41*, 1349–1352. [CrossRef]
68. Khattab, R.Y.; Arntfield, S.D. Functional properties of raw and processed canola meal. *LWT—Food Sci. Technol.* **2009**, *42*, 1119–1124. [CrossRef]
69. Shahidi, F.; Naczk, M.; Rubin, L.J.; Diosady, L.L. A Novel Processing Approach for Rapeseed and Mustard Seed-Removal of Undesirable Constituents by Methanol-Ammonia. *J. Food Prot.* **1988**, *51*, 743–749. [CrossRef]
70. Ghodsvali, A.; Khodaparast, M.H.H.; Vosoughi, M.; Diosady, L.L. Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. *Food Res. Int.* **2005**, *38*, 223–231. [CrossRef]
71. Deng, Y.; Dewil, R.; Appels, L.; Zhang, H.; Li, S.; Baeyens, J. The Need to Accurately Define and Measure the Properties of Particles. *Standards* **2021**, *1*, 4. [CrossRef]
72. White, N.D.G.; Jayas, D.S. Physical properties of canola and sunflower meal pellets. *Can. Biosyst. Eng.* **2001**, *43*, 349–352.
73. Kukelko, D.A.; Jayas, D.S.; White, N.D.G.; Britton, M.G. Physical Properties of Canola (Rapeseed) Meal. *Can. Agric. Eng.* **1988**, *30*, 61–64.
74. Manan, M.A.; Webb, C. Insights into physical characterization of solid state fermentation: From preliminary knowledge to practical application. *J. Biotech Res.* **2019**, *10*, 271–282.
75. Zhu, S.M.; Lin, S.L.; Ramaswamy, H.S.; Yu, Y.; Zhang, Q.T. Enhancement of Functional Properties of Rice Bran Proteins by High Pressure Treatment and Their Correlation with Surface Hydrophobicity. *Food Bioprocess Technol.* **2017**, *10*, 317–327. [CrossRef]
76. Shi, C.; He, J.; Yu, J.; Yu, B.; Mao, X.; Zheng, P.; Huang, Z.; Chen, D. Physicochemical Properties Analysis and Secretome of Aspergillus niger in Fermented Rapeseed Meal. *PLoS ONE* **2016**, *11*, e0153230. [CrossRef] [PubMed]
77. Multescu, M.; Marinas, I.C.; Susman, I.E.; Belc, N. Byproducts (Flour, Meals, and Groats) from the Vegetable Oil Industry as a Potential Source of Antioxidants. *Foods* **2022**, *11*, 253. [CrossRef] [PubMed]
78. Garside, P.; Wyeth, P. Identification of cellulosic fibres by FTIR spectroscopy: Differentiation of flax and hemp by polarized ATR FTIR. *Stud. Conserv.* **2006**, *51*, 205–211. [CrossRef]
79. Gao, Y.; Fangel, J.U.; Willats, W.G.T.; Vivier, M.A.; Moore, J.P. Dissecting the polysaccharide-rich grape cell wall changes during winemaking using combined high-throughput and fractionation methods. *Carbohydr. Polym.* **2015**, *133*, 567–577. [CrossRef]
80. Lucarini, M.; Durazzo, A.; Kiefer, J.; Santini, A.; Lombardi-Boccia, G.; Souto, E.; Romani, A.; Lampe, A.; Ferrari Nicoli, S.; Gabrielli, P.; et al. Grape Seeds: Chromatographic Profile of Fatty Acids and Phenolic Compounds and Qualitative Analysis by FTIR-ATR Spectroscopy. *Foods* **2019**, *9*, 10. [CrossRef]
81. Wang, L.P.; Shen, Q.R.; Yu, G.H.; Ran, W.; Xu, Y.C. Fate of biopolymers during rapeseed meal and wheat bran composting as studied by two-dimensional correlation spectroscopy in combination with multiple fluorescence labeling techniques. *Bioresour. Technol.* **2012**, *105*, 88–94. [CrossRef]
82. Heredia-Guerrero, J.A.; Beni tez, J.J.; Domi nguez, E.; Bayer, I.S.; Cingolani, R.; Athanassiou, A.; Heredia, A. Infrared and Raman spectroscopic features of plant cuticles: A review. *Front. Plant Sci.* **2014**, *5*, 305. [CrossRef]

83. Teh, S.S.; El-Din Bekhit, A.; Birch, J. Antioxidative polyphenols from defatted oilseed cakes: Effect of solvents. *Antioxidants* **2014**, *3*, 67–80. [CrossRef]
84. Nandasiri, R.; Eskin, N.A.M.; Thiyam-Höllander, U. Antioxidative Polyphenols of Canola Meal Extracted by High Pressure: Impact of Temperature and Solvents. *J. Food Sci.* **2019**, *84*, 3117–3128. [CrossRef]
85. Sepahpour, S.; Selamat, J.; Abdul Manap, M.; Khatib, A.; Abdull Razis, A. Comparative Analysis of Chemical Composition, Antioxidant Activity and Quantitative Characterization of Some Phenolic Compounds in Selected Herbs and Spices in Different Solvent Extraction Systems. *Molecules* **2018**, *23*, 402. [CrossRef] [PubMed]
86. Kralji, K.; Mamilovi, K. Influence of Cryogenic Grinding on the Nutritional and Antinutritional Components of Rapeseed Cake. *Appl. Sci.* **2023**, *13*, 5841. [CrossRef]

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Analysis of the Antioxidant and Antimicrobial Activity, Cytotoxic, and Anti-Migratory Properties of the Essential Oils Obtained from Cultivated Medicinal *Lamiaceae* Species

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Abstract

This study aims to highlight the therapeutic potential of some *Lamiacea* essential oils (EOs). For this purpose, eight EOs, including two from *Lavandula angustifolia* Mill. cultivated in Romania and Spain (LA1 and LA2), *Salvia officinalis* L. (SO), *Lavandula hybrida* Balb. ex Ging (LH), *Salvia sclarea* L. (SS), *Mentha smithiana* L. (MS), *Perovskia atriplicifolia* Benth. (PA), and *Mentha x piperita* L. (MP), were evaluated in vitro in terms of antioxidant, cytotoxic, antimicrobial, and anti-migratory activities. As regards the antioxidant capacity, expressed as the EO concentration that produces 50% of the maximum effect (IC_{50} value), the EOs obtained from the cultivated plants of the *Lamiaceae* family are ordered as follows: LA2 > LA1 > LH > MP > MS > SO > SS > PA. For the determination of antimicrobial activity, the reference strains used for testing were *Salmonella enterica* serotype *typhimurium*, *Shigella flexneri* serotype 2b, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Candida parapsilosis*. The most intense inhibitory effect was observed in EOs of MS and MP on all tested microbial strains. The cytotoxic and anti-migratory activity of EOs was tested on two melanoma cell lines (A375 and B164A5) and on a healthy keratinocyte line (HaCaT). EOs LA1 and MP manifested the highest selectivity on the analysed tumoural cells, by reducing their migration in comparison with the control, proving to have therapeutic potential.

Keywords: essential oil; *Lamiaceae*; antioxidant activity; antimicrobial activity; cytotoxic activity; anti-migratory activity

1. Introduction

According to the World Health Organization [1], more than 80% of the population prefers traditional medicine as the basis of any treatment. This alternative to conventional drugs might offer a lot of opportunities for researchers to study the plants that can be found in the vicinity of the cities where they work and take advantage of their medicinal properties. It has been observed that the essential oils resulting from their studies have beneficial properties with therapeutic uses [2–4].

Essential oils (EOs) are produced by plants as protection against pests and insects [5]. Many species from the *Lamiaceae* family are known to contain EOs used in the medical, cosmetic, and food industries [6]. EOs contain a mixture of active compounds, the most numerous of which are terpenoids and their oxygenated derivatives [7–9]. The chemical composition of EOs can be influenced by the geographical location of the plant species, the cultivation technology, and the conditioning and extraction method used [5]. They may also produce adverse or toxic effects on humans, as no rigorous tests are currently established to determine the safety of EO administration [5].

The pharmacological properties of EOs derived from *Lamiaceae* species have garnered significant attention due to their diverse biological activities, including antimicrobial, antioxidant, anti-inflammatory, and anticancer effects [6,10,11]. The genotoxicity of EOs has been evaluated several times over the years [12–16]. EOs from *Lamiaceae* species are rich in bioactive compounds, predominantly monoterpenes and sesquiterpenes, which contribute to their therapeutic properties [7–9]. These EOs are increasingly recognized for their potential applications in pharmaceuticals, food preservation, and cosmetics due to their ability to scavenge free radicals and mitigate oxidative stress. The antioxidant properties of *Lamiaceae* EOs are noteworthy. Research indicates that these oils can effectively scavenge free radicals, thereby mitigating oxidative stress, which is implicated in various chronic diseases [17,18].

The antioxidant activity of EOs is often attributed to their chemical composition, particularly the presence of phenolic compounds, known for their free-radical-scavenging abilities [7]. In addition, EOs from various species have demonstrated synergistic antioxidant effects when combined, indicating that the interactions between different EOs can enhance their overall efficacy [19]. The EOs from various *Lamiaceae* species exhibit varying degrees of antioxidant activity, suggesting that the specific phytochemical profiles of these oils play a crucial role in their antioxidant effectiveness [20]. The total phenolic content of EOs has been correlated with their antioxidant activity, with higher total phenolic content values often leading to enhanced free-radical-scavenging capabilities [21,22]. For example, the EOs of *Lavandula angustifolia* have been shown to exhibit strong antioxidant activity, which is attributed to its high content of phenolic compounds [9]. Research also indicates that EOs can serve as natural alternatives to synthetic antioxidants in food products, effectively prolonging shelf life and maintaining quality by preventing oxidative deterioration [23,24].

The antimicrobial and antimutagenic activities of EOs belonging to the *Lamiaceae* family underlie many applications, including pharmaceutical, medicinal, cosmetic, and even processed foods applications, due to the bioactive compounds contained in them [12,25]. Studies have shown that EOs from various species of the *Lamiaceae* family possess potent antifungal properties, particularly against *Candida albicans* and *Staphylococcus aureus* [26,27]. In addition, *Lamiaceae* EOs have demonstrated anti-inflammatory effects, which can be beneficial in treating inflammatory conditions [8,28]. These oils have been explored for their potential in wound healing and tissue regeneration, further underscoring their therapeutic versatility [28,29].

The application of *Lamiaceae* EOs extends beyond traditional medicine into modern pharmaceutical and agricultural industries. Their insecticidal properties have been documented, showing efficacy against agricultural pests [29,30]. This suggests that these oils could serve as natural alternatives to synthetic pesticides, promoting sustainable agricultural practices.

In the context of the above, the present research aims at investigating potential therapeutic uses of some *Lamiaceae* EOs. For this purpose, tests regarding the antioxidant, antimicrobial, cytotoxic, and anti-migratory activity on carcinogenic cells were performed using eight EOs from cultivated plants belonging to the *Lamiaceae* family, including two from *Lavandula angustifolia* Mill. (LA1 cultivated in Romania and LA2 cultivated in Spain), *Salvia officinalis* L. (SO), *Lavandula hybrida* Balb. ex Ging (LH), *Salvia sclarea* L. (SS), *Mentha smithiana* L. (MS), *Perovskia atriplicifolia* Benth. (PA), and *Mentha x piperita* L. (MP), respectively.

The results obtained proved the potential therapeutic capacity of the analysed EOs from antioxidant, antimicrobial, and antitumoural perspectives, confirming the previous results in the literature [5,12,14,16–18,20]. This fact opens the opportunity for more complex and detailed investigations.

2. Results

2.1. DPPH Radical-Scavenging Assay of EOs Obtained from Cultivated Medicinal Species Belonging to the Lamiaceae Family

The antioxidant activity of the eight samples of EOs obtained from cultivated species of the *Lamiaceae* family are expressed using IC_{50} values, which are detailed in Table 1. The IC_{50} value represents the half-maximal inhibitory concentration of each EO, where 50% of its maximal effect is observed, which refers to the potency required to obtain a 50% antioxidant effect.

Table 1. The antioxidant capacity [%] of the EOs obtained from cultivated *Lamiaceae* medicinal species, as compared with standard (ascorbic acid), and the corresponding IC_{50} values, respectively.

Sample No.	Sample Code	Antioxidant Capacity [%]	$IC_{50} \pm$ SD [μg/mL]
1	LA1	88.85 ± 0.024	15.84 ± 0.92
2	LA2	90.90 ± 0.002	13.28 ± 0.67
3	SO	55.56 ± 0.187	94.73 ± 1.18
4	LH	83.81 ± 0.004	19.39 ± 0.98
5	SS	52.05 ± 0.079	109.28 ± 1.34
6	MS	70.02 ± 0.117	65.78 ± 1.21
7	PA	38.81 ± 0.041	186.84 ± 1.56
8	MP	89.18 ± 0.003	45.75 ± 1.06
9	Ascorbic acid	95.92 ± 0.026	0.7 ± 0.05

The antioxidant capacity percentage obtained for all the EO samples represents an average of three measurements ± standard deviation (SD). Further, by linear regression analysis, the IC_{50} value was calculated. It can be observed that all the analysed samples show an antioxidant capacity from moderate to high, in some cases (*Lavandula angustifolia* Mill. and *Lavandula hybrida* Balb. ex Ging). Therefore, regarding the concentration tested (200 μg/mL), the antioxidant potential of each essential oil is ordered as follows: LA2 > LA1 > LH > MP > MS > SO > SS > PA.

2.2. Antimicrobial Action of the Analysed EOs

2.2.1. Analysis of the Diameters of Inhibition Zones Obtained by the Disc Diffusion Method

Table 2 shows the zones of inhibition for the analysed EOs. The zones were obtained by the disc-diffusion method. For all bacterial strains, a Gentamicin micro-tablet of 10 μg, except 120 μg for the *Enterococcus faecalis* strain, was used as a control. For fungal strains of *Candida*, a 10 μg Fluconazole micro-tablet was used as a control.

Table 2. The diameters of the inhibition zones obtained by the disc-diffusion method (mm).

EO	*K. pneumoniae*	*S. flexneri*	*S. enterica*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. faecalis*	*C. albicans*	*C. parapsilosis*
MS	21	20	22	22	15	21	22	33	33
SO	6	9	9	9	6	10	9	10	10
LA1	13	14	10	14	6	20	19	30	30
PA	6	6	6	10	6	20	16	20	19
LH	6	6	9	10	6	21	20	20	21
MP	25	20	20	26	21	26	24	20	20
LA2	10	11	9	9	6	16	15	19	18
SS	6	6	6	6	6	22	20	20	20

EOs with an inhibition zone diameter greater than 15 mm were considered to have antimicrobial activity and were further tested by the dilution method.

For the SO oil, no antimicrobial activity was recorded, while LA1, PA, LH, LH, LA2, and SS oils were active only on Gram-positive cocci and fungi. The other EOs (MS and MP) had inhibitory activity on all reference strains tested.

Table 3 presents the matrix of the Euclidean distances calculated for the measured diameters of the paired samples of EOs used for the determination of similarities in antimicrobial activity.

Table 3. Matrix of the Euclidean distances (Source: authors' own calculations using PAST 4.03).

EO	MS	SO	LA1	PA	LH	MP	LA2	SS
MS	0.00							
SO	45.97	0.00						
LA1	20.42	33.17	0.00					
PA	36.11	18.68	19.36	0.00				
LH	33.65	21.75	17.69	5.48	0.00			
MP	20.95	42.40	30.17	36.54	34.66	0.00		
LA2	33.41	15.39	18.47	8.37	10.10	33.57	0.00	
SS	36.84	22.18	19.95	6.08	5.20	37.55	11.18	0.00

The dendrogram constructed using Euclidean distances with paired groups has a cophenetic correlation coefficient with a value of 0.91, indicating that, to a large extent, the dendrogram keeps the distances in agreement with the empirical data. The EOs LH and SS have close activity in size. Also, even PA and LA2 have similar activities. Important differences are observed between MS and MP, each of which is relative to SO. The groups formed are represented in Figure 1.

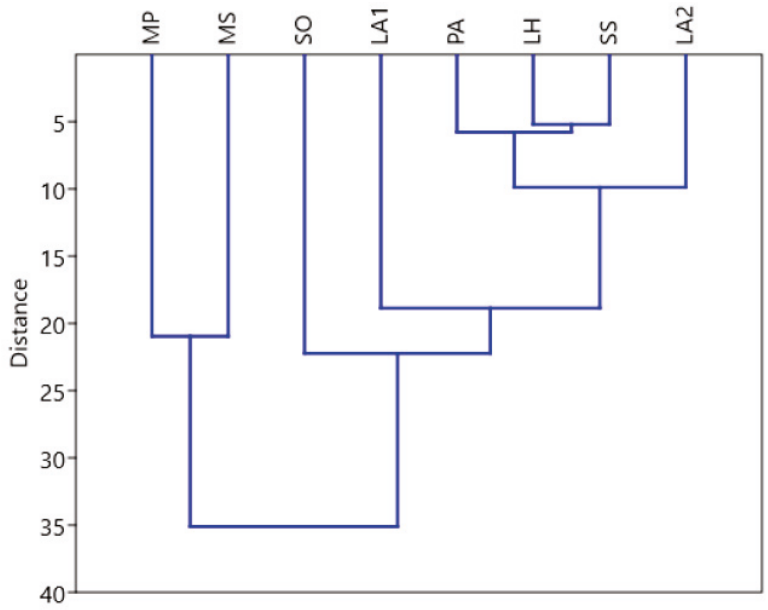


Figure 1. Hierarchical cluster for describing similarity in the activity of the studied plant samples (Source: authors' own graphical representation of experimental data using PAST 4.03).

The mean values corresponding to the EOs studied then formed the basis for prioritising the sample types. Together with other statistical indicators, the data are presented in the boxplot in Figure 2.

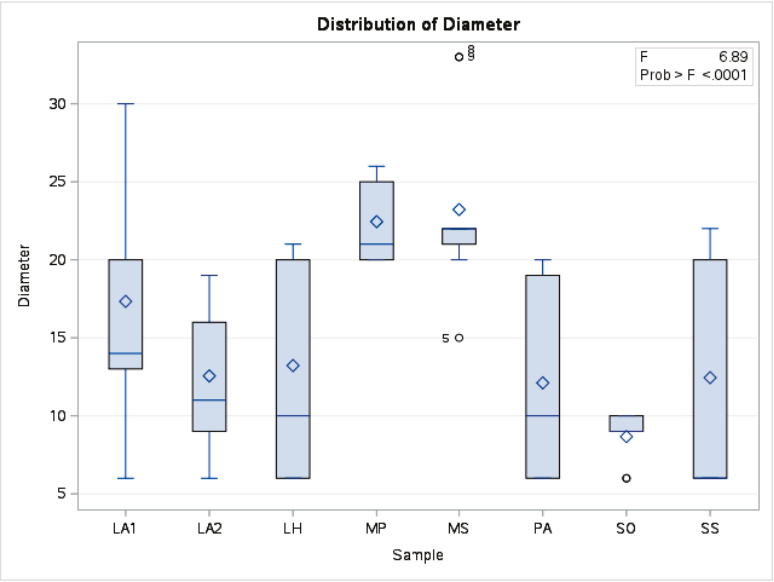


Figure 2. Boxplot distribution of measured diameter values for the analysed EO samples (Source: authors' own graphical representation of experimental data using SAS Studio).

2.2.2. Determination of Minimum Inhibitory Concentration and Minimum Concentration

The EO/DMSO dilutions were as follows: 40, 20, 10, 5, and 2.5 mg/mL. The values for the MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration)/MFC (minimum fungicidal concentration) are given in Table 4.

Table 4. MIC and MBC/MFC values obtained by the macro-dilution method.

EO	*K. pneumoniae*		*S. flexneri*		*S. enterica*		*E. coli*		*P. aeruginosa*		*S. aureus*		*E. faecalis*		*C. albicans*		*C. parapsilosis*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
MS	10	20	10	20	10	20	10	20	20	40	10	10	10	10	5	5	5	5
LA1											10	20	10	20	5	5	5	5
PA											10	20	10	20	10	10	10	10
LH											10	20	10	20	10	10	10	10
MP	10	20	10	20	10	20	10	20	20	40	5	10	10	10	10	10	10	10
LA2											10	20	20	20	10	10	10	10
SS											20	20	20	20	10	10	10	10

The EO extracted from LA1 exhibits antimicrobial activity against Gram + cocci (*S. aureus* 20 mm and *E. faecalis* 19 mm) and fungi (*C. albicans* 30 mm and *C. parapsilosis* 30 mm). The main chemical compounds of the LA EO are linalool (22.11%) and linalyl acetate (20.384%) in approximately equal proportions.

LH and SS exhibited congruent antimicrobial activity with an inhibition diameter of about 20–22 mm for Gram + cocci (*S. aureus* and *E. faecalis*) and fungi (*C. albicans* and *C. parapsilosis*).

The main chemical compounds of the LH EO are linalool (35.86%) and eucalyptol (17.84%). As for the SS strain, linalyl acetate and linalool are found in 80%, which supports the antimicrobial activity of these compounds.

The EO extracted from LA2 is found to have weaker antimicrobial activity on Gram + cocci (*S. aureus* 16 mm and *E. faecalis* 15 mm) and fungi (*C. albicans* 19 mm and *C. parapsilosis* 18 mm) compared to the EOs extracted from LH and SS.

The MS EO exhibited antibacterial and antifungal activity on all strains tested, with a maximum zone of inhibition on fungi (33 mm *C. albicans* and *C. parapsilosis*).

The EO extracted from the strain of SO had a diameter of the zone of inhibition of less than 15 mm. Thus, it was considered to have no antimicrobial activity and was not further tested by the dilution method.

The major compound of the EO extracted from SO is beta-thujone (16.84%), which does not seem to induce antibacterial and antifungal activity on the tested species (*K. pneumoniae*, *S. flexneri*, *S. enterica*, *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, *C. albicans*, *C. parapsilosis*).

2.3. Antitumoural Effects of the EOs

2.3.1. Analysis of the Cytotoxic Effect of the EOs

In this part of the study, the effect of the EOs was analysed on two melanoma cell lines and on a keratinocyte line at 24 h post-stimulation. Two concentrations (50 and 150 µg/mL) of each sample were tested. All calculations were based on the solvent used to prepare the solutions—dimethylsulfoxide (DMSO). The highest DMSO concentration tested was 0.15%.

Figure 3 shows the viability of A375 human melanoma cells after stimulation with the EOs. At the lowest dose tested, 50 µg/mL, the samples had no significant effect on cell viability. The graph shows a slight reduction in tumour cell viability when stimulated with the EOs obtained from LA2 and SO.

Increasing the dose used (150 µg/mL) led to a decrease in the viability of A375 tumour cells following stimulation with certain samples (Figure 3). The most significant result was observed in the case of the SO EO sample (*** $p < 0.001$). The viability of tumour cells also decreased in the PA EO sample (*** $p < 0.001$) and to a lesser extent in the LA1 EO sample (** $p < 0.01$). By increasing the dose, a greater decrease in cell viability was observed for the LA1 sample compared to LA2, but without statistical significance, which is different from the results obtained for the same samples tested at a low dose.

In the case of the MP EO sample, a decrease in cell viability (87.1 ± 7.5% versus the solvent DMSO 0.15%) was observed. The other three EO samples (LH, SS, and MS) at this dose had no effect on human melanoma cells.

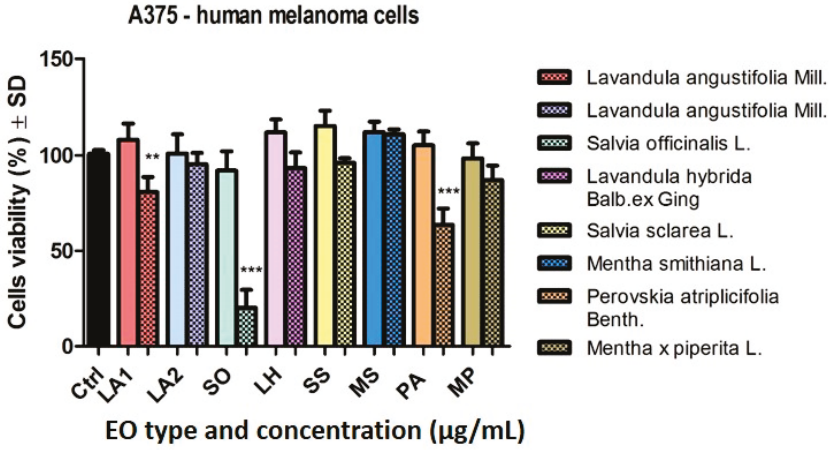


Figure 3. A375 human melanoma cell viability, after stimulation with the EOs (50 and 150 µg/mL—clear vs. dotted texture of the colour fill) for 24 h. Data are expressed as mean ± SD (*** $p < 0.001$; ** $p < 0.01$). The comparison between groups was performed using the One-way ANOVA test followed by Dunnett's post-test.

The effect of the aforementioned samples was also evaluated on the murine melanoma cell line B164A5 (Figure 4). At the low dose tested (50 µg/mL), the EO sample from PA reduced tumour cell viability (81.91 ± 3.6% vs. DMSO). The MP EO also showed a slight reduction in cell viability. The other samples had no major influence at 50 µg/mL on this cell line.

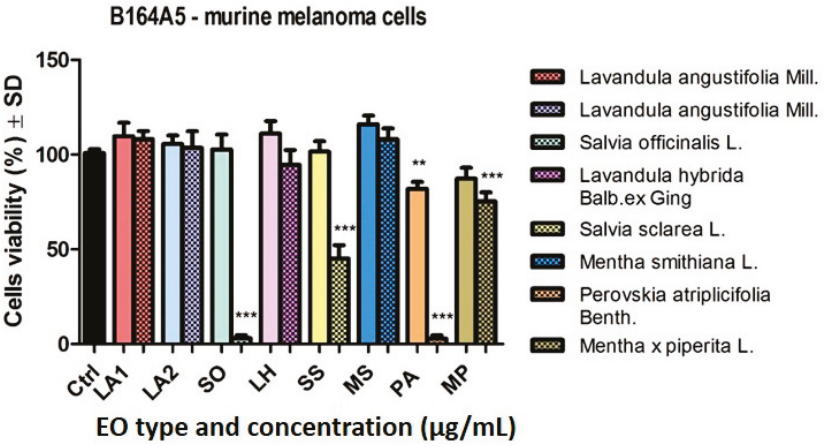


Figure 4. B164A5 murine melanoma cell viability, after stimulation with the EOs (50 and 150 µg/mL—clear vs. dotted texture of the colour fill) for 24 h. Data are expressed as mean ± SD (*** $p < 0.001$; ** $p < 0.01$). The comparison between groups was performed using the One-way ANOVA test followed by Dunnett's post-test.

By increasing the dose used (150 µg/mL) (Figure 4), there was a significant reduction in cell viability after stimulation with the EO samples of SO and PA (*** $p < 0.001$). On B164A4 cells, it was observed that stimulation with EO samples of SS and MP led to a decrease in murine melanoma cell line viability, whereas at the same dose, these samples had no significant effect on the human melanoma cell line.

The effect of EOs was also tested on a non-tumour line, namely, HaCaT keratinocytes. At 50 µg/mL, the samples did not affect significantly cell viability (Figure 5).

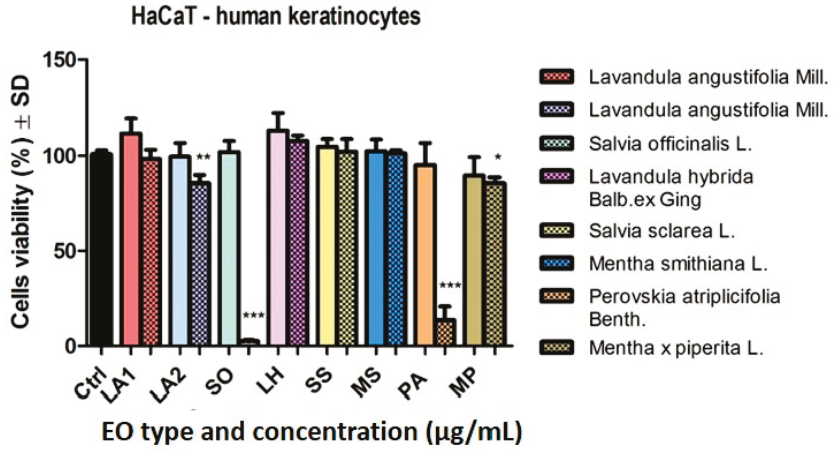


Figure 5. HaCaT human keratinocyte viability after stimulation with the EOs (50 and 150 µg/mL—clear vs. dotted texture of the colour fill) for 24 h. Data are expressed as mean ± SD (*** $p < 0.001$; ** $p < 0.01$ and * $p < 0.050$). The comparison between groups was performed using the One-way ANOVA test followed by Dunnett's post-test.

At the highest dose tested, the EOs of SO and PA produced a significant decrease (*** $p < 0.001$) in cell viability. The EOs of LA2 (** $p < 0.01$) and MP (* $p < 0.05$) also elicited a reduction in keratinocyte viability (Figure 5).

2.3.2. Analysis of the Anti-Migratory Effect of the EOs Using the Scratch Technique

Tumour cells exhibit an increased ability to migrate and this property has an important role in tumour progression.

In this experiment, the anti-migratory effect of EOs was determined on two melanoma cell lines and on a keratinocyte line. The lowest dose (50 µg/mL) of each sample was tested, as it is desired to assess whether a reduction in the migratory ability of tumour cells occurs.

Determination of the Anti-Migratory Effect of the EOs on A375 Human Melanoma Cell Line

In Figures 6 and 7, the effect of the EOs belonging to the *Lamiaceae* family is shown. The images were compared with a control (untreated cells grown in culture medium) and DMSO (cells stimulated with the solvent used to obtain stock solutions) at the same concentration.

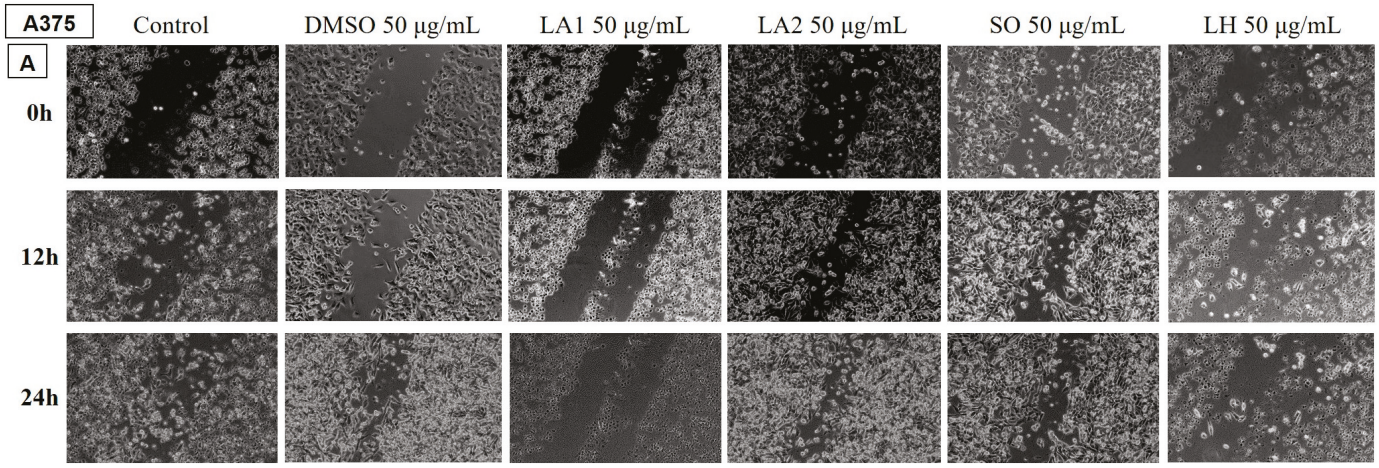


Figure 6. The anti-migratory effect of the tested EOs (50 µg/mL) (A—LA1; LA2; SO and LH) on human melanoma cell line A375. The images were taken at 0, 12, and 24 h after stimulation.

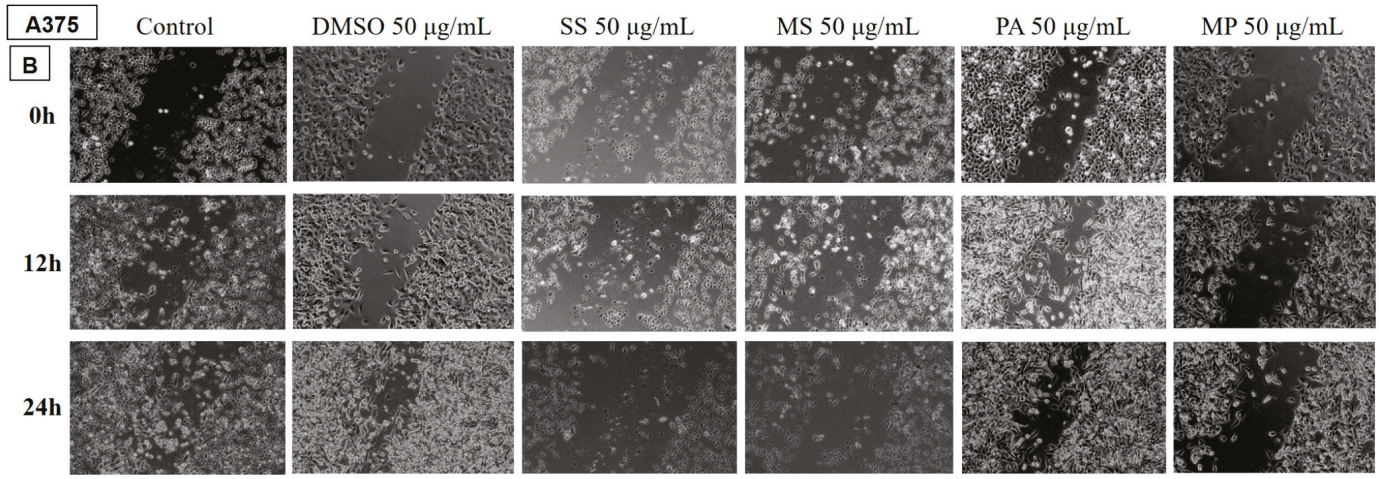


Figure 7. The anti-migratory effect of the tested EOs (50 µg/mL) (B—SS; MS; PA and MP) on human melanoma cell line A375. The images were taken at 0, 12, and 24 h after stimulation.

The tested samples reduced the migration of A375 tumour cells compared to the control and DMSO groups, but to a lesser extent for the EOs of LA2, SO, and PA.

Figure 6 shows the effect of LA1 on A375 melanoma cells. It can be seen that at the dose of 50 µg/mL, the sample reduces the migratory ability of tumour cells compared to the control and DMSO.

On the other hand, the 50 µg/mL EO samples of LA2, SO, and PA do not inhibit the migratory capacity of the human melanoma cell line, highlighting that at this dose, they have no antitumour effect.

All other tested samples, including LH, SS, MS, and MP, produced a reduction in tumour cell migration compared to the control and DMSO groups, proving to have beneficial properties against melanoma.

Determination of the Anti-Migratory Effect of the EOs on B164A5 Murine Melanoma Cell Line

The effect of the EOs obtained from different species belonging to the *Lamiaceae* family was also tested on the B164A5 murine melanoma cell line (Figures 8 and 9). The images were compared with the control and DMSO at the same concentration. Again, the lower dose of 50 µg/mL was tested for all samples.

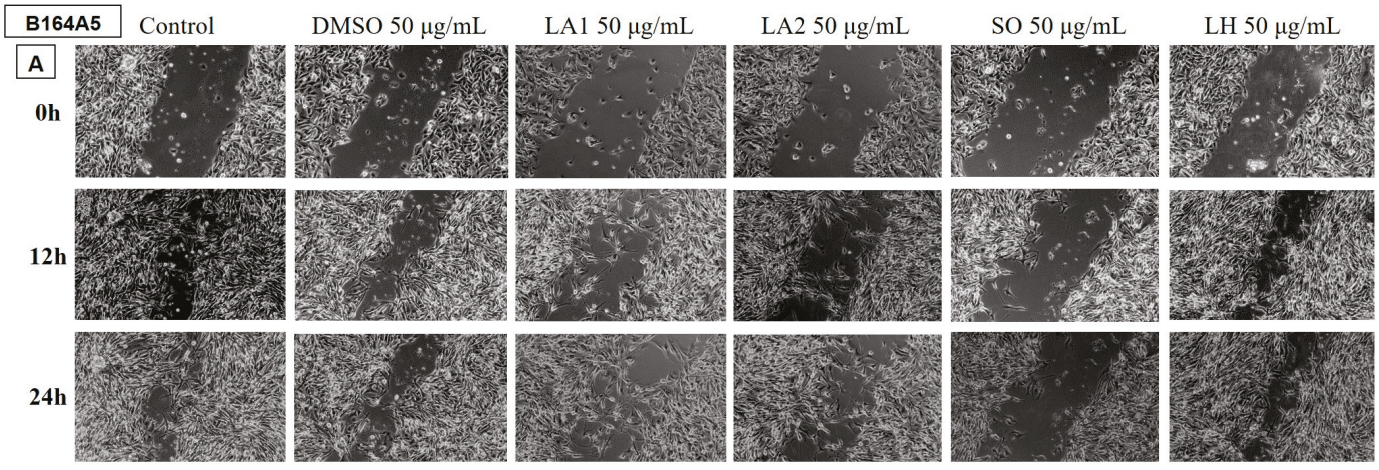


Figure 8. The anti-migratory effect of the tested EOs (50 µg/mL) (A—LA1; LA2; SO and LH) on murine melanoma cell line B164A4. The images were taken at 0, 12, and 24 h after stimulation.

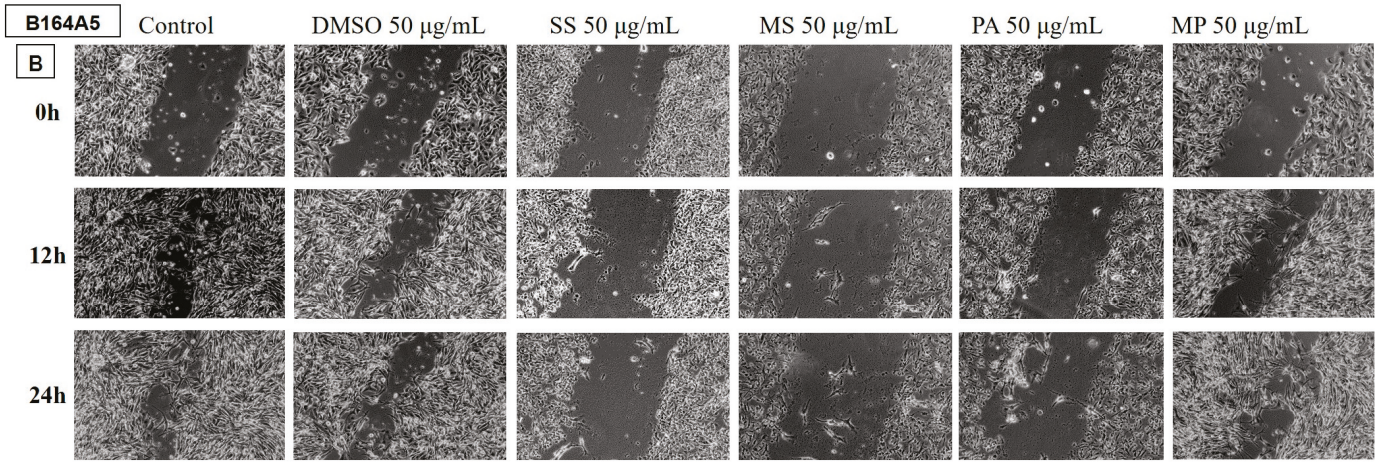


Figure 9. The anti-migratory effect of the tested EOs (50 µg/mL) (B—SS; MS; PA and MP) on murine melanoma cell line B164A4. The images were taken at 0, 12, and 24 h after stimulation.

The LH and MP EO samples did not have an anti-migratory effect on the tumour cells, the migration rate for these samples being similar to the control.

The rest of the tested samples significantly reduced the migration ability of murine melanoma cells.

Determination of the Anti-Migratory Effect of the EOs on HaCaT, Human Keratinocyte

The EOs (50 µg/mL) were also tested on a non-tumour cell line, HaCaT human keratinocyte (Figures 10 and 11), to assess whether it showed selectivity, i.e., whether it affected only tumour cells and did not affect the migratory capacity of healthy cells. The images were compared with the control and DMSO at the same concentration.

The obtained data showed that the EO samples of LA1, LA2, PA, and MP stimulated the migration of human keratinocytes, similar to the control group, while the rest of the tested EO samples reduced the migration capacity of HaCaT cells.

In comparison with the data obtained on the human melanoma cell line, it can be found that the EOs of LA1 and MP reduced the migration capacity of tumour cells and, at the same dose, stimulated the migration of human keratinocytes, proving to be selective samples with beneficial effects against human melanoma.

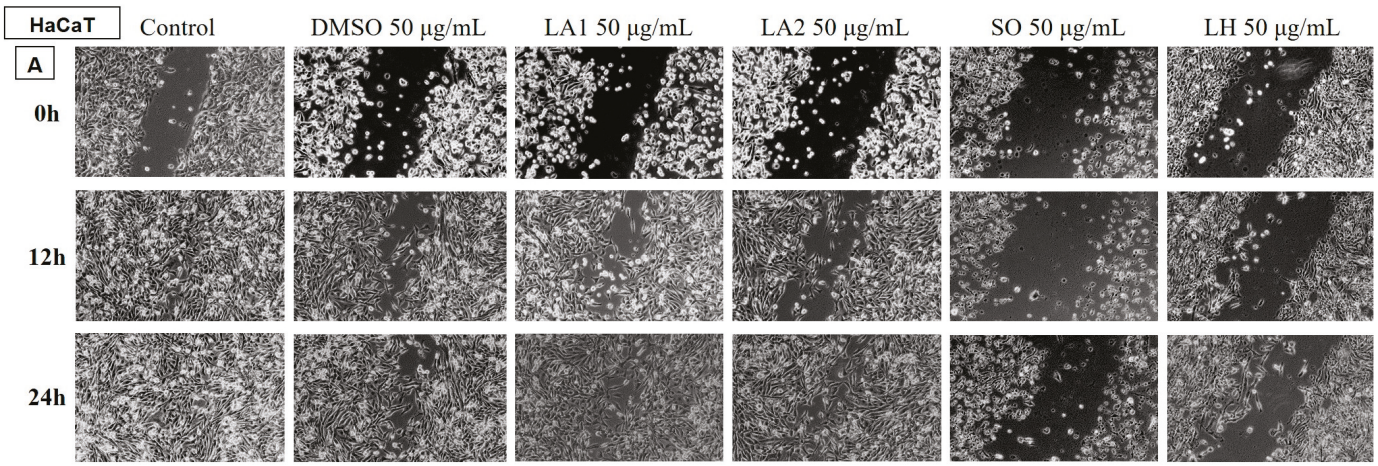


Figure 10. The anti-migratory effect of the tested EOs (50 µg/mL) (A—LA1; LA2; SO and LH) on the HaCaT cell line, keratinocytes. The images were taken at 0, 12 and 24 h after stimulation.

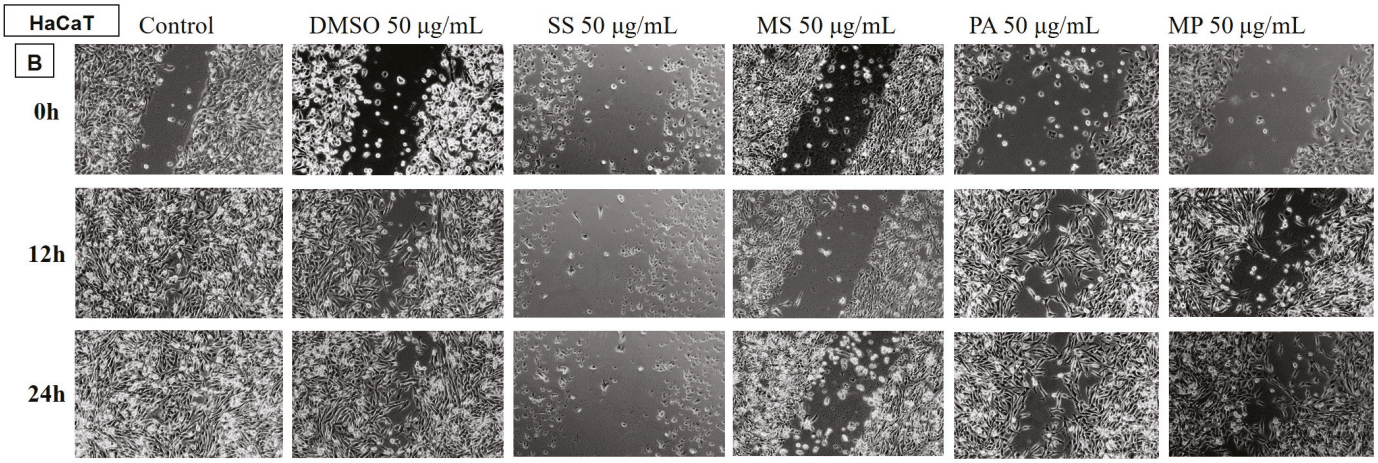


Figure 11. The anti-migratory effect of the tested EOs (50 µg/mL) (B—SS; MS; PA and MP) on the HaCaT keratinocyte cell line. The images were taken at 0, 12, and 24 h after stimulation.

3. Discussion

3.1. Antioxidant Capacity of EO

The antioxidant capacity refers to the ability of a substance to neutralize free radicals and mitigate oxidative stress, which is linked to various chronic diseases and ageing processes. Oxidative stress is considered to be an imbalance in redox homeostasis between the excessive production of reactive oxygen species (ROS) and a deterioration in antioxidant capacity. This can occur in various diseases such as cancer, diabetes, vascular and brain dysfunction, ageing, etc. ROS are a diverse group of highly reactive molecules that play critical roles in various biological processes. Their reactivity is primarily attributed to the presence of unpaired electrons in their molecular structure, which makes them capable of engaging in chemical reactions that can lead to oxidative stress and cellular damage. ROS can be classified into two main categories: free radicals, which possess unpaired electrons, and non-radical species, such as hydrogen peroxide (H_2O_2) [31]. The most common free radicals include superoxide (O_2^-) and hydroxyl radicals (·OH), both of which are known for their high reactivity and ability to oxidize a wide range of biological macromolecules, including lipids, proteins, and DNA [32]. The generation of ROS occurs as a natural by-product of aerobic metabolism, particularly during mitochondrial respiration, where electrons are transferred through the electron transport chain [33,34]. Under normal physiological conditions, ROS are produced in controlled amounts and are kept in balance by antioxidant systems, which include enzymes like superoxide dismutase and catalase, as well as small molecules like glutathione [32]. However, when the production of ROS exceeds the capacity of these antioxidant defences, it leads to oxidative stress [35,36]. The reactivity of ROS is not solely detrimental; they also serve important signalling functions within cells. Low concentrations of ROS can act as second messengers in various signalling pathways, influencing processes such as cell growth, differentiation, and immune responses [37]. This dual role of ROS—acting as both signalling molecules and potential agents of damage—highlights their complexity in biological systems. The balance between their beneficial and harmful effects is crucial for maintaining cellular homeostasis and responding to environmental stresses [38].

The antioxidant capacity is typically quantified using several standardized assays that measure the effectiveness of antioxidants in different contexts, including biological systems and food products. The properties of EOs have been most widely evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, which measures the ability of these oils to donate hydrogen and neutralize free radicals [39,40]. In the present research, we

analysed the antioxidant capacity of eight EOs obtained from cultivated *Lamiaceae* species by the classical DPPH method. Our results showed a moderately high antioxidant capacity, expressed in terms of IC_{50} values, for the EOs analysed. For instance, the EOs from *Lavandula angustifolia* Mill (LA1 and LA2) exhibit the highest IC_{50} value (15.84 ± 0.92 μg/mL and 13.28 ± 0.67 μg/mL). Our outcomes are comparable with those reported in the literature [41–44], highlighting the significant biological activities of these EOs, particularly in antioxidant and antimicrobial capacities. The differences between our results and those reported in the literature [41–44] came from the variability in the chemical composition of *Lavandula angustifolia* EOs, which has also been documented [45,46].

The EO of *Lavandula hybrida* is characterized by a rich profile of terpenes, predominantly linalool and linalyl acetate, which are known for their ability to neutralize free radicals, thereby enhancing the oil's overall antioxidant capacity [47]. The presence of phenolic compounds in EOs has also been linked to antioxidant activity, further supporting the potential of *Lavandula hybrida* in this regard [48]. In comparative studies, the antioxidant capacity of *Lavandula hybrida* has been evaluated alongside other species [42,43,49,50]. Our findings revealed an IC_{50} value of 19.39 ± 0.98 μg/mL, which indicates, as in other research studies, the ability of the *Lavandula hybrida* EO to scavenge free radicals, although specific IC_{50} values were not detailed in the findings in [51].

The EO from *Salvia officinalis* has shown varying IC_{50} values across different studies. It has been stated that the antioxidant properties of the *S. officinalis* EO are often attributed to the presence of phenolic compounds and terpenes, which are prevalent in the oil's composition [52,53]. Our findings revealed an IC_{50} value of 94.73 ± 1.18 μg/mL for the EO of *Salvia officinalis* L. and 109.28 ± 1.34 μg/mL for the EO of *Salvia sclarea* L. In the case of *Salvia officinalis* EOs, the antioxidant properties are often attributed to the presence of phenolic compounds and terpenes, which are prevalent in the oil's composition [52–54]. The variability regarding the results is due to the *Salvia officinalis* EOs' chemical composition, which depends on factors such as geographical location and extraction methods; these factors can influence the observed IC_{50} values and overall efficacy of the essential oil [55,56]. The antioxidant capacity of the *Salvia sclarea* L. essential oil has been investigated in various studies, revealing significant insights into its bioactive properties [57–60]. In the study reported by Aćimović et al., the essential oil of *S. sclarea* exhibited an IC_{50} value of approximately 400 μg AAE/mL in the DPPH assay, indicating its capacity to neutralize free radicals [61]. This value, while higher than that obtained in the present study (109.28 ± 1.34 μg/mL), still demonstrates a notable antioxidant potential of *S. sclarea* EO.

While specific IC_{50} values for *Mentha smithiana* essential oil were not directly reported in the literature, related studies on other *Mentha* species provide a comparative context, indicating that its IC_{50} values can vary widely depending on the methodology used [62–65]. The results of the present study regarding the *Mentha* species' EO agree with the literature and are even better [66–69]. We obtained an IC_{50} value of 45.75 ± 1.06 μg/mL for the EO obtained from the *Mentha piperita* cultivated medicinal plant and an IC_{50} value of 65.78 ± 1.21 μg/mL for the EO from *Mentha smithiana* cultivated medicinal plant.

The EOs from *Perovskia atriplicifolia* Benth. have been shown to contain a diverse array of phytochemicals, primarily monoterpenes and sesquiterpenes, which contribute to their bioactivity [70,71]. Although specific IC_{50} values for *Perovskia atriplicifolia* EOs were not detailed in the available literature, related studies on other species within the *Lamiaceae* family suggest that EOs from this group typically exhibit strong antioxidant properties [71,72]. Our findings revealed an IC_{50} value of 186.84 ± 1.56 μg/mL for the EO of the *Perovskia atriplicifolia* cultivated medicinal plant, a value which is comparable with the literature [71,72]. Overall, the antioxidant activity of EOs is often linked to their

chemical constituents. The variability in antioxidant capacity is influenced by the chemical composition of the EOs, which is affected in turn by environmental factors.

3.2. Antimicrobial Effect of EO

Our results proved that the LA1 EO had antibacterial properties against Gram + cocci *S. aureus* and *E. faecalis* and antifungal activity against *C. albicans* and *C. parapsilosis*.

In the literature, it is mentioned that the LA EO is effective in the inhibition and control of bacterial strains and could be used as a natural antibacterial agent [73]. Our results showed a low antimicrobial activity on Gram + cocci and fungi compared to *Lavandula hybrida*. The results obtained are in agreement with the literature data, as published by de Rapper et al. [74]; the complete chemical profile of the EO extracted from *Lavandula angustifolia* Mill coincides with our results. Moreover, it was demonstrated that the lavender EO exhibits significant antibacterial activity against various pathogens, which is crucial for its application in food safety and medicinal products [75]. The EO's effectiveness in inhibiting bacterial growth is attributed to its major components, including linalool and linalyl acetate, which possess inherent antimicrobial properties [76,77]. In addition, studies have shown that *Lavandula hybrida* exhibits varying inhibitory concentration levels against different microorganisms [51,78,79].

LH and SS EOs manifested antibacterial activity for Gram + cocci (*S. aureus* and *E. faecalis*) and antifungal activity (*C. albicans* and *C. parapsilosis*), due to their chemical composition [53,59]. The antimicrobial efficacy of the *Salvia officinalis* essential oil has also been extensively studied [80,81]. As regards the EO of *S. sclarea*, studies have reported MIC values against *Staphylococcus aureus* and *Staphylococcus epidermidis* as low as 5.0 mg/mL [82,83]. Additionally, the essential oil has shown effectiveness against multidrug-resistant strains, further emphasizing its potential as a natural antimicrobial agent [82,84].

The antimicrobial properties of *Mentha* EOs have been extensively documented. The essential oil from *Mentha piperita* exhibited significant antimicrobial activity, with MIC values reported as low as 3.75 μL/mL against various pathogens [85]. In our research, the MS EO demonstrated antimicrobial properties on all pathogenic strains tested, both bacterial and fungal, according to other reported studies [86–91]. The synergistic effects of the *Mentha piperita* essential oil with conventional antifungal agents have also been explored, suggesting that combining these oils with existing treatments could enhance their efficacy and reduce the likelihood of resistance [92]. Therefore, all the findings reported underscore the potential of *Mentha piperita* EOs as effective antimicrobial agents, with specific IC_{50} values that vary based on the concentration and target microorganism. The ongoing investigation into the chemical composition and environmental influences of these oils will further elucidate their practical applications in medicine and agriculture.

The results obtained in our study show that the SO EO has no antimicrobial activity on the tested bacterial and fungal strains. In other research, the antimicrobial activity of the endemic population of *Salvia officinalis* from Egypt, with a major composition of camphor (25.1%), a-thujone (22.2%), and b-thujone (17.7%), was tested by chemical analysis. It was found to have antimicrobial activity against *K. pneumoniae*, *S. aureus*, *E. coli*, and *C. albicans*, while no effect was found against *P. aeruginosa* [93].

As regards the *Perovskia atriplicifolia* medicinal plant investigated, the EOs have demonstrated promising antimicrobial activities against various pathogens. The presence of active compounds in the EOs suggests potential applications in both food preservation and therapeutic contexts. EOs from related species have been noted for their effectiveness against *Escherichia coli* and *Staphylococcus aureus*, which are common bacterial pathogens [94,95]. The IC_{50} values for these activities are crucial for determining the efficacy of these oils in

clinical and agricultural applications, although specific values for *Perovskia atriplicifolia* remain to be fully elucidated.

3.3. Cytotoxic and Anti-Migratory Effects of the EOs

The results obtained indicate that the EO samples with the highest cytotoxic activity are *Salvia officinalis* L. (SO) and *Perovskia atripicifolia* Benth. (PA). The EOs analysed show a dose-dependent cytotoxic effect; the most significant data are obtained after 24 h cell stimulation, with a dose of 150 μg/mL. These samples do not show selectivity against tumour cells because, at high doses, they also reduced keratinocyte viability.

In terms of anti-migratory capacity, the obtained data indicate that two EO samples, LA1 and MP, reduced the migration capacity of melanoma to a higher extent in comparison with the control. A comparative evaluation of the results obtained on the human melanoma cell line and human keratinocytes showed that the LA1 and MP samples showed increased selectivity; they stimulated the migration of healthy cells and inhibited the migration capacity of tumour cells, respectively.

Different types of extracts and EOs obtained from plants belonging to the *Lamiaceae* family are frequently studied for their antitumour effect, showing positive effects against melanoma [96,97]. Cocan et al. [97] evaluated the biological activity of an ethanolic extractive solution obtained from *Salvia officinalis* L. The authors determined the effect of the extractive solution (at concentrations of 50 and 100 μg/mL) on A375 human melanoma cell lines and the B164A5 murine melanoma cell line. All samples produced a significant dose-dependent decrease in tumour viability.

In another experiment, the EOs obtained from *Salvia officinalis* L. and *Thymus vulgaris* L. species were tested for antifungal and antiproliferative activity on two melanoma cell lines, A375 and B164A5 [98]. The EO obtained from *Salvia officinalis* L. produced a significant inhibition of tumour proliferation; at a dose of 100 μg/mL, it inhibited proliferation by 50.5% for the murine melanoma line and by 47.5% for the human melanoma line. The *Thymus vulgaris* L. EO inhibited melanoma cell proliferation but to a lesser extent. Regarding the chemical composition, the EO from *Salvia officinalis* L. had caryophyllene, camphene, eucalyptol, and β-pinen as its main compounds [98].

Our results in terms of viability are in agreement with those obtained by Alexa et al. [98]; the EO obtained from *Salvia officinalis* L. (SO) produced a significant decrease in cell viability at the high dose tested (150 μg/mL) in both tumour cell lines. On the other hand, the chemical composition of the EO was different in our case, with the EO from SO having D-limonene, beta-thujone, and alpha-thujone as its main compounds.

Our findings show that the EOs with high antioxidant capacity had better antimicrobial effects; this relationship should be further investigated, as it is useful in the selection of EOs with therapeutic potential. The results obtained on the antimicrobial effect of the EOs obtained from *Lamiaceae* species can represent therapeutic alternatives with natural bioactive compounds that may be effective in the treatment of some bacterial and fungal infections resistant to synthetic substances.

Some of the analysed EOs have inhibited the proliferation and migration of melanoma cell lines, and similar results are present in the literature. This path can be approached in future research for the identification of the bioactive compounds from the EOs and other factors that can influence the therapeutic potential.

4. Materials and Methods

The analysed EOs were extracted from medicinal plants cultivated in two geographical locations, the Young Naturalists Station Timisoara and the town of Rivas in Spain during the period 2013–2016. The plants come from organic crops and were harvested

during the flowering period at midday. The species used for the essential oil extraction belongs to the *Lamiaceae* family, including *Lavandula angustifolia* Mill. (LA1—cultivated at Timișoara), *Lavandula angustifolia* Mill. (LA2—cultivated in Rivas), *Salvia officinalis* L. (SO), *Lavandula hybrida* Balb. ex Ging (LH), *Salvia sclarea* L. (SS), *Mentha smithiana* L. (MS), *Perovskia atriplicifolia* Benth. (PA), and *Mentha x piperita* L. (MP).

The collected plants were identified and stored in the Department of Medicinal Plants, University of Life Sciences "King Mihai I" from Timisoara, except the LA2 sample, which was processed and analysed in Spain. Each plant received a voucher number.

4.1. Materials Used

4.1.1. EO Extraction and Composition Analysis

For the medicinal plant species studied, the amount of essential oil obtained from 100 g of the dried herb was determined and the drying yield was calculated. The extraction of the EOs was performed with a Clevenger Apparatus by steam distillation (Table 5). The amount of volatile oil obtained from each species was determined, and the drying yield was also calculated, as shown in Table 5.

Table 5. Average drying efficiency and the amount of essential oil obtained from the studied species.

Sample Code	Fresh *herba* (g)	Dry *herba* (g)	Drying Efficiency (%)	EO from Sample (mL)	EO ml/100 g Dry *herba*
LA1	3450	1100	31.88	15	1.36
LA2	2968	1012	29.33	18	1.78
SO	2843	987	33.25	4.6	0.47
LH	3526	1188	33.69	20.1	1.69
SS	2487	795	31.96	10.1	1.27
MS	2507	890	35.5	5.5	0.61
PA	2717	918	33.78	8.3	0.9
MP	11700	3630	31.02	40.5	1.11

The composition of EOs was determined by the chromatographic method and by a GC-MS analysis of the samples at the Laboratory of "Aurel Vlaicu" University of Arad, Romania; the results are presented in Tables A1–A8 from Appendix A. The EO constituents were determined based on their mass spectra using the NIST 14 library [99] and the Wiley 09 library [100].

4.1.2. Reagents

The determination of antioxidant activity was carried out with ethyl alcohol 96% (*v*/*v*), which was purchased from Chemical Company SA, Iasi, Romania, ascorbic acid, which was purchased from Lach-Ner (Neratovice, Czech Republic), and DPPH (TBF5255V), which was purchased from Sigma Aldrich (St. Louis, MI, USA). For the determination of microbial activity, DMSO was used as a solvent for the samples purchased from Sigma Aldrich. Gentamicin and Fluconazole micro-tablets were obtained from Bio-Rad, Marnes-la-Coquette (France). Agar Columbia +5% sheep blood and Sabouraud with chloramphenicol, respectively, were purchased from bioMerieux (Marcy-l'Étoile, France).

4.1.3. Microbial Strains

The reference strains used (Table 6) for testing were selected to represent microbial species that can colonise the intestinal tract.

Table 6. Reference strains.

Microbial Species	ATCC	Manufacturer
Salmonella enterica serotype *typhimurium*	14028	Thermo Scientific (Waltham, MA, USA)
Shigella flexneri serotype 2b	12022	
Enterococcus faecalis	51299	
Escherichia coli	25922	
Klebsiella pneumoniae	700603	
Pseudomonas aeruginosa	27853	
Staphylococcus aureus	25923	
Candida albicans	10231	
Candida parapsilosis	22019	

4.2. Methods Used

4.2.1. DPPH Radical-Scavenging Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is commonly used to determine the antioxidant activity of different types of extracts/samples [101]. The experimental method is based on the ability to reduce dark purple DPPH in the presence of an antioxidant to a pale-yellow compound. A 1 mM solution of DPPH in 96% (*v*/*v*) ethyl alcohol was prepared by weighing 19.7 mg of DPPH dissolved in 50 mL of 96% EtOH. This solution was kept refrigerated in brown glass throughout the analysis. In parallel, a 2 mM solution of ascorbic acid in 96% ethanol was prepared by weighing 0.4 mg of ascorbic acid in one mL of the 96% EtOH solution. The ethanolic solution of ascorbic acid was considered the positive standard for the samples to be analysed.

According to a previously reported method, modified and developed by our research group [102], the antioxidant capacity of the eight samples of EOs was established using the DPPH free-radical-scavenging assay. Briefly, 0.5 mL of the sample solution to be analysed, 0.5 mL of the 1 mM DPPH alcohol solution, and 2 mL of the solvent (96% ethanol) are introduced into a 4 mL cuvette. The absorbance of the solutions is determined continuously at a wavelength of 516 nm for 1200 s using a T70 UV/VIS spectrophotometer (PG Instruments Ltd., Wibtoft, Lutterworth, United Kingdom). The same procedure is applied for the ascorbic acid solution: 0.5 mL of the 2 mM ascorbic acid solution is mixed with 0.5 mL of the 1 mM DPPH alcohol solution and 2 mL of the solvent (96% ethanol).

The antioxidant activity was calculated using the following formula:

$$\mathrm{AOA}(\%) = \left(\frac{\mathrm{A_{DPPH}} - \mathrm{A_{EO}}}{\mathrm{A_{DPPH}}}\right) \times 100 \quad (1)$$

where the variables are defined as follows:

AOA = is the antioxidant activity of the test samples analysed (%);

$\mathrm{A_{EO}}$ = is the absorbance of each EO test sample in the presence of the DPPH free radical measured at 516 nm;

$\mathrm{A_{DPPH}}$ = is the absorbance of the DPPH free radical measured at a wavelength of 516 nm without the EO test sample.

4.2.2. Determination of Antimicrobial Activity

A. Disc-diffusion assay

The determination of antimicrobial activity was carried out both by the standardised disc-diffusion method (disc-diffusion susceptibility) and by the dilution method with the determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC). The disc-

diffusion method, being easier and cheaper to perform, has been used for screening the antimicrobial activity of EOs. The detailed work protocol is presented in Appendix B. According to the literature, a diameter $\geq$ 15 mm is considered highly susceptible [103].

B. Macro-dilution method

The macro-dilution technique allows for the determination of MIC and CMB/CMF.

The work principle applied was based on increasing dilutions of the antibacterial substance in tubes of liquid medium. Fixed amounts of the microbial culture are then added and incubated for 24 h at 37 °C, and the lowest concentration of antibacterial substance tested (essential oil) that does not allow bacterial growth is aimed for [104]. Also, the detailed working protocol can be seen in Appendix B.

C. Determination of CMB/CMF

Using a sterile disposable loop, 1 µL from each tube, including the control, was seeded on Columbia agar +5% sheep blood or Sabouraud with chloramphenicol and incubated 24 h at 37 °C, and then the highest dilution (lowest concentration) at which germs did not grow was read, representing the CMB/CMF [105].

4.2.3. Determination of the Antitumour Activity

To determine the cytotoxic and anti-migratory activity of the EOs obtained from *Lamiaceae* species, two melanoma cell lines (A375 and B164A5) and a healthy keratinocyte cell line (HaCaT) were used. To achieve this objective, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis [97] was performed to determine cell viability after stimulation with the EOs, and a Scratch analysis [106,107] was performed to determine the anti-migratory effect of the EOs.

The A375 human melanoma cell line was purchased from the American Type Culture Collection (ATCC CRL-1619™) and B164A5 murine melanoma cells were purchased from Sigma-Aldrich (Munich, Germany). HaCaT human keratinocytes were provided by the Department of Dermatology, University of Debrecen, Hungary.

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM 4.5 g/l glucose; Sigma-Aldrich, Taufkirchen, Germany), supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, Taufkirchen, Germany) and antibiotics to avoid contamination (1% penicillin/streptomycin; Sigma-Aldrich, Taufkirchen, Germany). Cell growth was performed in a humidity-controlled atmosphere with 5% CO_2 at 37 °C. For cell number determination, the Neubauer chamber was used in the presence of Trypan blue.

A. MTT assay

Cells were cultured in 96-well plates at a density of 1 × 104 cells/well and allowed to adhere to the well base overnight. Samples were applied in two concentrations (50 and 150 µg/mL) and incubated for 24 h with the cells. The MTT reagent—10 µL of 5 mg/mL MTT solution (Sigma-Aldrich, Budapest, Hungary) was added to each well (volume in the well was 100 µL). Intact mitochondrial reductase transformed and precipitated the MTT solution as blue crystals after a 3 h contact period.

Precipitated crystals were dissolved in 100 µL of the lysis solution (Sigma-Aldrich). Finally, samples were spectrophotometrically analysed at 570 using a microplate reader (xMark Microplate Spectrophotometer, Bio-Rad, Tokyo, Japan). The DMSO solution was used to prepare stock solutions for the samples tested [108].

B. Scratch assay

The migratory capacity of tumours (A375 and B164A5) and cells, respectively, was determined using the Scratch assay. The protocol was applied as previously described in the literature [109]. A number of 2 × 10^5 cells/well were cultured in 12-well plates for

48 h before the experiment. A sterile pipette tip was used to draw a line in well-defined areas of the wells (at 80–90% confluence). Cells that detached as a result of the procedure were removed by washing with phosphate-buffered saline (PBS, Thermo Fisher Scientific, Cambridge, MA, USA). Cells were then stimulated with the lowest sample concentration (50 μg/mL). Images of cells in the culture were taken at the beginning of the experiment (0 h after stimulation) and at 12 and 24 h using the Olympus IX73 inverted microscope (Olympus, Tokyo, Japan) [107,109].

4.2.4. Statistical Methods

Statistical calculations referring to the antimicrobial and antioxidant activity of the analysed EOs were performed using PAST 4.03 [110] and SAS Studio [111]. The evaluation of similarity and distances was performed using hierarchical clustering and the associated dendrogram. The assessment of differences between groups delimited by the application of a sample type was performed using ANOVA. Pairwise comparisons between groups were performed using Tukey's pairwise test.

The results regarding the antitumour activity of the studied EOs were expressed as mean ± standard deviation. The comparison between groups was performed using One-way ANOVA, followed by Dunnett post-test. A p-value of ≤ 0.05 was considered to have statistical significance. Analyses were performed using GraphPad Prism 5 [112].

The Euclidean distances calculated for the measured diameters of the paired samples of the oils used were the basis of an analysis to determine similarities in their antimicrobial action. The formula used is $d = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2}$, where x_i represents the diameter values determined for the first plant sample type and y_i represents the diameter values determined for the second sample type.

5. Conclusions

The EOs from *Lamiaceae* species exhibit a wide array of pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, and anticancer activities. The rich chemical composition of these oils, characterized by various bioactive compounds, underpins their therapeutic potential. In the present study, we showed that the volatile oils obtained from plants belonging to the *Lamiaceae* family induced a significant antioxidant activity, the results being close to the value of ascorbic acid, which was used as a standard, especially in the case of *Lavandula* species. The results of this study confirm the traditional use of these species as antioxidants and suggest that the species cultivated in western Romania possess a high antioxidant capacity due to the polyphenols contained in the plants.

The EOs of *Lavandula angustifolia* Mill. (LA1), *Perovskia atripicifolia* Benth. (PA), *Lavandula hybrida* Balb. ex Ging (LH), *Lavandula angustifolia* Mill. (LA2), and *Salvia sclarea* L. (SS) were active only on Gram-positive cocci and fungi. The other EOs, *Mentha smithiana* L. (MS) and *Mentha x piperita* L. (MP), had inhibitory activity on all the reference strains tested. The EO of *Mentha smithiana* L. (MS) shows antibacterial and antifungal activity on all strains tested, with a maximum zone of inhibition on the fungi *C. albicans* and *C. parapsilosis*. For the EO of *Salvia officinalis* L. (SO), no antibacterial activity was recorded, while the EO of *Perovskia atriplicifolia* Benth. (PA) was active only on Gram-positive cocci and fungi.

From the perspective of cytotoxic activity, the obtained results indicated that the samples with the highest efficiency were *Salvia officinalis* L. (SO) and *Perovskia atripicifolia* Benth. (PA). The analysed EOs show a dose-dependent cytotoxic effect. These samples do not show selectivity against tumour cells because, at high doses, they also reduced keratinocyte viability.

In terms of anti-migratory capacity, the data obtained indicate that many samples reduced the migratory capacity of melanoma cell lines. The comparative evaluation of the results obtained on the human melanoma line and human keratinocytes indicated that LA1 and MP had the most potent effect by inhibiting the migration ability of tumour cells and stimulating HaCaT migration. The EO samples showed increased selectivity; they stimulated the migration of healthy cells and inhibited the migratory capacity of tumour cells.

EOs are bio-compounds with many potential applications and uses in pharmaceutics. This direction shall be deepened due to the high demand for effective and affordable therapeutical solutions. Future research should focus on elucidating the mechanisms of action of these oils and exploring their applications in both clinical and industrial settings.

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

Appendix A

Table A1. Chemical composition of the essential oil of *Lavandula angustifolia* Mill. (LA1).

Chemical Compound	%	RT (min)
Eucalyptol	2.144	
Trans-Beta-Ocimene	5.976	10.836
cis-beta-Ocimene	2.477	14.876
Linalool	22.11	17.589
endo-Borneol	2.23	18.043
Terpinen-4-ol	3.811	18.59
Alpha-Terpineol	2.13	21.202
Linalyl acetate	20.384	22.22
Lavandulyl acetate	7.599	26.35
Carvacrol	3.333	27.255
Diphenhydramine	7.31	28.742
cis-beta-Farnesene	3.86	31.771
tau-Cadinol	2.473	

Table A2. Chemical composition of the essential oil of *Lavandula angustifolia* Mill. (LA2).

Chemical Compound	%	RT (min)
Alpha-Thujene	0.12	5.656
Alpha-Pinene	0.38	5.893
Camphene	0.33	6.379
Sabinene	0.6	7.487
Beta-Pinene	0.51	8.075
n-Hexyl acetate	0.2	8.908
3-Carene	0.26	9.139
p-Cymene	0.13	9.643
Benzene, 1-methyl-3-(1-methylethyl)-	0.84	9.861
Eucalyptol	1.45	10.126
D-Limonene	1.02	10.193
trans-beta-Ocimene	2.18	10.819
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	0.85	11.577
Sabinene hydrate	0.49	13.177
Linalool	30.15	15.322
(+)-2-Bornanone	1.57	16.321
endo-Borneol	2.46	18.258
(−)-4-Terpineol	7.19	18.47
Alpha-Terpineol	1.83	19.243
Borneol, acetate	0.24	19.983
cis-(−)-1,2-Epoxy-p-menth-8-ene	0.16	20.228
Linalyl acetate	23.71	21.207
(Z)-Geraniol	0.81	21.605
Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	0.39	22.004
Lavandulol acetate	6.21	22.251
Hexyl tiglate	0.07	23.491
(R)-lavandulyl (R)-2-methylbutanoate	0.59	24.482
cis-Geranyl acetate	0.9	25.03
Hexanoic acid, hexyl ester	0.23	25.15
(−)-beta-Bourbonene	0.09	25.35
Sesquithujene	0.1	25.497
Beta-Curcumene	0.05	25.934
Alpha-Cedrene	0.1	26.183
Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	4.79	26.361
cis-alpha-Bergamotene	0.2	26.768
Aromandendrene	0.12	27.005
Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	2.54	27.26
Hexadecane	0.21	27.59
Germacrene D	0.19	28.001
(R)-lavandulyl (R)-2-methylbutanoate	0.05	28.51
Alpha-Guaiene	0.12	28.596
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	1.07	28.853
delta-Cadinene	0.18	29.003
Bicyclo[2.2.1]hept-2-ene, 1,7,7-trimethyl-	0.07	29.165
1-Methyl-6-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0]heptane	0.1	29.792
Spiro[tricyclo[3.3.1.1(3,7)]decane-2,2′-oxetan]-4′-one, 3′-methylene-	0.16	30.155
Caryophyllene oxide	2.63	30.589
(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	0.1	31.239
Epicubenol	0.12	31.488
tau-Cadinol	1.06	32.165
Muurol-5-en-4-one <cis-14-nor->	0.08	33.115

Table A3. Chemical composition of the essential oil of *Salvia officinalis* L. (SO).

Chemical Compound	%	RT (min)
5-Undecene, 7-ethenyl-	0.32	3.832
3-Decyne	0.07	4.013
Alpha-Thujene	0.22	5.663
1R-alpha-Pinene	2.59	5.908
Camphene	2.17	6.385
Sabinen	9	7.488
Beta-Pinene	0.34	8.083
Eucalyptol	1.04	9.913
D-Limonene	15.04	10.209
trans-beta-Ocimene	0.15	10.843
Sabinene hydrate	0.12	13.119
Beta-Thujone	16.84	14.713
Alpha-Thujone	8.81	15.247
(+)-2-Bornanone	3.83	16.317
trans-Sabinol	0.2	16.87
L-Pinocarveol	0.37	17.127
L-Borneol	6.8	18.259
trans-Ocimenol	0.37	19.211
(−)-Myrtenol	0.19	19.523
Pentanoic acid, 4-hexen-1-yl ester	0.17	20.305
Linalyl acetate	0.05	21.075
L-bornyl acetate	0.73	21.995
Sabinyl isobutanoate	0.06	22.261
Alpha-Cubebene	0.07	24.308
Alpha-ylangene	0.04	24.977
Alpha-Copaene	0.12	25.114
(−)-beta-Bourbonene	0.09	25.347
Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	0.07	25.96
Isocaryophyllene	6.33	26.375
Humulene	5.33	27.321
Gamma-Muurolene	0.24	27.859
(+)-Ledene	0.06	28.376
Alpha-Cadinene	0.06	28.51
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1alpha,4a.beta.,8a.alpha.)-	0.11	28.853
Delta-Cadinene	0.18	29.033
1-Methyl-6-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0]heptane	0.27	29.796
Caryophyllene oxide	3.61	30.599
Viridiflorol	7.4	31.057
(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	2.6	31.262
Copalol	0.2	31.751
Caryophylla-4(12),8(13)-dien-5alpha-ol	0.2	32.188
cis-alpha-Bisabolene	0.06	35.543
Cyclopentadecanone	0.15	36.394
Butyl 6,9,12,15-octadecatetraenoate	0.37	36.737
Isopimara-9(11),15-diene	0.2	38.46
n-Hexadecanoic acid	0.15	40.968
Epimanool	2.46	42.17
(+)-Valencene	0.15	42.631

Table A4. Chemical composition of the essential oil of *Lavandula hybrida* Balb. ex Ging (LH).

Chemical Compound	%	RT (min)
Alpha-Pinene	1.14	5.983
Camphene	0.45	6.43
Beta-Ocimene	1.85	7.491
Beta-Pinene	1.07	8.092
Eucalyptol	17.84	10.116
Limonene	2.01	10.225
trans-beta-Ocimene	5.42	10.748
Beta-Ocimene	1.91	11.487
Gamma-Terpinene	0.47	13.847
Linalool	35.86	14.633
(+)-2-Bornanone	6.06	15.902
endo-Borneol	6.8	17.478
Terpinen-4-ol	3.62	17.906
Alpha-Terpineol	1.98	18.473
Linalyl acetate	5.41	20.977
Lavandulol acetate	0.86	22.084
Caryophyllene	1.04	26.247
cis-beta-Farnesene	4.19	27.192
Germacrene D	0.74	27.859
(R)-lavandulyl (R)-2-methylbutanoate	0.42	28.435
Alpha-Bisabolol	0.86	32.678

Table A5. Chemical composition of the essential oil of *Salvia sclarea* L. (SS).

Chemical Compound	%	RT (min)
Beta-Pinene	0.52	8.102
Beta-Ocimene	0.65	11.484
Linalool	10.12	14.553
Alpha-Terpineol	1.15	18.483
Linalyl formate	1.01	19.463
Linalyl acetate	69.41	21.099
Neryl acetate	0.69	24.32
Lavandulol acetate	1.09	24.856
Alpha-Cubebene	0.71	25.074
Aromandendrene	3.23	26.252
Germacrene D	8.61	27.87
(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene	1.56	28.271
Alpha-Farnesene	0.43	28.503
Alloaromadendrene	0.82	30.819

Table A6. Chemical composition of the essential oil of Mentha *smithiana* L. (MS).

Chemical Compound	%	RT (min)
Camphenol, 6-	0.048	9.498
Tricyclenne	0.006	6.942
A-phellandrene	0.03	9.425
A-pinene	1.009	5.983
Camphene	0.223	6.43
Sabinene	0.912	8.200
B-pinene	1.515	7.747
Limonene	14.18	10.225
Eucalyptol	0.56	9.913
1,3,6 octatriene 3,7 dimethyl (Z) (beta cis ocimene)	0.071	11.577
A-terpinene	0.029	6.726
P-mentha-1,4(8) diene	0.084	10.98
Linalool	0.336	15.322
P-menth-1-en-8-ol	0.064	18.573
Geraniol acetate	0.256	23.70
B-caryophilene	2.12	26.824
B-farnesene	0.763	26.33

Table A7. Chemical composition of the essential oil of *Perovskia atriplicifolia* Benth. (PA).

Chemical Compound	%	RT (min)
Phellandrene	0.14	7.85
2-hexanal	0.03	4.97
Trans-sabinene hydrate	0.19	12.523
borneol	4.79	28.833
camphene	1.45	6.707
sabinene	1.02	6.91
P-cymene	0.32	9.748
Beta-myrcene	0.53	6,98
1,8-cineole	5.85	10.105
3-carene	7,56	9.226
Alpha-terpinene	0.25	6.726
o-cymene	0.38	10.30
Limonene	0.49	10.250
Cis-ocimene	0.75	10.82
Beta-ocimeneY	0.57	8.59
y-terpinene	0.46	9.78
Dehydro-p-cymene	0.03	11.09
Alpha-terpinolene	0.04	10.98
Cis--sabinene hydrate	0.06	10.20
linalool	5.21	19.548
Iso-amyl isovalerate	0.03	11.60
camphor	2.12	18.787
4-terpineol	0.42	17.93
Cumyl alcohol	0.04	2.429
Alfa-terpinol	0.65	18.573
methylcyclohexane	0.07	2.61
calarene	0.98	19.95
valeranone	2.34	35.74
Alpha-thujene	0.07	5,40
Bornyl formate	0.44	17.36
Beta-Pinene	0.98	7.04
Alpha-Pinene	4.30	5.85

Table A8. Chemical composition of the essential oil of *Mentha x piperita* L. (MP).

Chemical Compound	%	RT (min)
Alpha-Pinene	0.93	5.994
3-Octanone	0.75	7.324
Beta-Pinene	1.38	7.51
Beta-Myrcene	0.7	8.114
Eucalyptol	10.26	10.129
D-Limonene	9.7	10.272
Trans-beta-Ocimene	0.94	10.755
Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.beta.,5.alpha.)-	4.43	12.57
Alpha-Terpineol	0.31	18.508
8-p-Menthen-2-ol	5.58	18.749
D-Carvone	42.28	20.207
Borane carbonyl	0.68	20.495
8-p-Menthen-2-yl, acetate, trans	1.28	23.256
2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, acetate, (1R-cis)-	0.57	24.306
Jasmone	0.48	25.06
Beta-Bourbonene	1.83	25.298
Beta-Elemene	0.85	25.485
Caryophyllene	4.94	26.269
Isogermacrene D	0.17	26.91
Bicyclosesquiphellandrene	0.34	26.962
Beta-copaene	0.68	27.395
Germacrene D	8.47	27.887
Gamma-Elemene	1.65	28.286
Alpha-Selinene	0.8	28.516

Appendix B

Determination of antimicrobial activity (detailed)

A. Disc-diffusion assay

The determination of antimicrobial activity was carried out both by the standardised disc-diffusion method (disc-diffusion susceptibility) and by the dilution method, with the determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC).

The disc-diffusion method, being easier and cheaper to perform, has been used for screening the antimicrobial activity of EOs.

Principle of the method used: The antimicrobial substance (essential oil) is deposited on the surface of an agar culture medium, which has been pre-seeded with the test bacteria. Two phenomena occur simultaneously: the diffusion of the oil into the medium and multiplication of the micro-organism. In areas where the oil achieves concentrations higher than the MIC, bacterial growth no longer occurs. The circumference of the inhibition zone is established from the first hours of incubation as the geometric location of the points where the oil has reached the MIC at the critical time of culture. Thus, the diameter of the inhibition zone varies inversely with the MIC.

The composition of the culture medium, its pH, inoculum density, stability and diffusion of the test substance (oil), and incubation time and temperature, are all variables that influence the results of the disc-diffusion method.

Materials needed:

1. Reference strains were seeded on Columbia agar +5% sheep blood and Sabouraud with fungal chloramphenicol, respectively, with 24 h thermostatting at 37 °C. The

inoculum density, i.e., the number of bacteria brought into contact with the tested oil, is an important element and condition for the reproducibility of the results. According to the CLSI [Clinical and Laboratory Standards Institute] standard, a microbial suspension in sterile saline equivalent to 0.5 Mc Farland (108 CFU/mL) is prepared [103].

2. For the culture medium, we used Mueller-Hinton agar (bioMerieux, Marcy-l'Étoile, France), which is recommended by CLSI. For the Candida strains, we used Mueller-Hinton medium supplemented with methylene blue. The sterility control of the media consisted of incubating a plate from the batch used for 24 h at 37 °C.
3. An unimpregnated microcompressed blank, 6 mm in diameter (BioMaxima, Lublin, Poland), was used.
4. Other materials used include sterile saline, cotton wool pads on wooden rods, and tweezers for the deposition of microcompresses.

The technique of the disc-diffusion antibiogram is as follows: standardized bacterial suspension was obtained by suspending colonies from the 24 h culture on Columbia agar +5% sheep blood and Sabouraud medium with chloramphenicol for *Candida* strains, respectively, in sterile saline.

Seeding was carried out with a sterile cotton swab, which was dipped into the suspension; excess was removed by pressing against the walls of the tube and passing over the surface of the medium in three directions until the entire surface was covered, with a circular marginal streak being made to obtain a uniform inoculum.

After inoculation, the plates were left for 5–10 min at room temperature to facilitate inoculum uptake into the medium, after which the microcompresses were deposited with a minimum distance of 25 mm between discs and 15 mm from the edge of the Petri dish. From the undiluted test oils, 10 µL per tablet was deposited.

For each oil, a single plate was used, on which the micro-tablet with the oil and two control micro-tablets were deposited. The positive control consisted of a Gentamicin micro-tablet of 10 µg and 120 µg, respectively, for the *Enterococcus faecalis* strain. For *Candida* strains, the positive control was a 10 µg Fluconazole micro-tablet. An unimpregnated micro-tablet was used as a negative control. The plates thus prepared were incubated for 24 h at 35 ± 2 °C.

The interpretation of results was as follows: after thermostatting, readings were taken by measuring the diameters of the inhibition zones with a ruler. According to the literature, a diameter ≥ 15 mm is considered highly susceptible [104].

For quality control, all tests were performed in duplicate.

B. Macro-dilution method

The macro-dilution technique allows for the determination of MIC and CMB/CMF.

The work principle is as follows: increasing dilutions of the antibacterial substance are made in tubes of the liquid medium. Fixed amounts of the microbial culture are then added and incubated for 24 h at 37 °C, and the lowest concentration of the antibacterial substance tested (essential oil) that does not allow bacterial growth is aimed for [103].

The materials required are as follows: sterile test tubes, liquid culture medium (Mueller-Hinton broth), test oils, 0.5 Mc Farland microbial suspension from reference strains, pipettes, and a thermostat.

The working method is as follows: An inoculum of approximately 5×10^5 CFU/mL is prepared from the 0.5 Mc Farland bacterial suspension. This can be achieved by diluting the 0.5 McFarland suspension to a ratio of 1:150, resulting in a suspension of 10^6 CFU/mL. Further dilution at a ratio of 1:2 will bring the final inoculum to 5×10^5 CFU/mL [103].

Testing was carried out in five test tubes as follows: 0.5 mL of the 5×10^5 CFU/mL bacterial suspension was pipetted into each test tube; then, 0.4 mL of Mueller-Hinton broth

and 0.1 mL of the essential oil dilution was obtained in DMSO (1/2, 1/4, 1/8, 1/16, 1/32), resulting in a volume of 1 mL, which was homogenised. For each strain tested, a positive control containing 0.5 mL of the bacterial suspension, 0.4 mL of Mueller-Hinton broth, and 0.1 mL of DMSO was used to check the growth of the strain. The negative control contained 0.1 mL of oil and 0.4 mL of Mueller-Hinton broth +0.5 mL of DMSO.

For the interpretation, first, the control is read, which should be turbid (if the germ has grown). If there is no bacterial growth, the broth in the control tube should remain clear, invalidating the test. If the blank is adequate, the reaction has been performed correctly and the reading is taken from the tubes in which the medium has remained unchanged (the germ has not grown because it is inhibited by the oil). The lowest concentration at which no germ growth has occurred is the CMI.

C. Determination of CMB/CMF:

Using a sterile disposable loop, 1 µL from each tube, including the control, was seeded on Columbia agar +5% sheep blood or Sabouraud with chloramphenicol and incubated 24 h at 37 °C; then, the highest dilution (lowest concentration) at which germs did not grow was read, representing the CMB/CMF [105].

References

1. Integrating Traditional Medicine in Health Care. Available online: https://www.who.int/southeastasia/news/feature-stories/detail/integrating-traditional-medicine (accessed on 17 October 2024).
2. Khan, M.; Kihara, M.; Omoloso, A.D. Antimicrobial Activity of the Alkaloidal Constituents of the Root Bark of Eupomatia Laurina. *Pharm. Biol.* **2008**, *41*, 277–280. [CrossRef]
3. Pavrez, M.; Mahboob, H.K.; Zahuul, I.; Shek, M.H. Antimicrobial Activities of the Petroleum Ether, Methanol and Acetone Extracts of Kaempferia Galangal. Rhizome. *J. Life Earth Sci.* **2005**, *1*, 25–29.
4. Parthasarathy, S.; Bin Azizi, J.; Ramanathan, S.; Ismail, S.; Sasidharan, S.; Said, M.I.M.; Mansor, S.M. Evaluation of Antioxidant and Antibacterial Activities of Aqueous, Methanolic and Alkaloid Extracts from *Mitragyna Speciosa* (Rubiaceae Family) Leaves. *Molecules* **2009**, *14*, 3964–3974. [CrossRef]
5. Stringaro, A.; Colone, M.; Angiolella, L. Antioxidant, Antifungal, Antibiofilm, and Cytotoxic Activities of *Mentha* spp. Essential Oils. *Medicines* **2018**, *5*, 112. [CrossRef] [PubMed]
6. Mamadalieva, N.Z.; Akramov, D.K.; Ovidi, E.; Tiezzi, A.; Nahar, L.; Azimova, S.S.; Sarker, S.D. Aromatic Medicinal Plants of the *Lamiaceae* Family from Uzbekistan: Ethnopharmacology, Essential Oils Composition, and Biological Activities. *Medicines* **2017**, *4*, 8. [CrossRef]
7. Isnaini, N.; Annisa, A.; Prajaputra, V.; Maryam, S.; Idroes, R.; Khairan, K. Chemical Composition and Biological Activities of Essential Oils in the Family *Lamiaceae*. *IOP Conf. Ser. Earth Environ. Sci.* **2024**, *1356*, 012097. [CrossRef]
8. Galovičová, L.; Borotová, P.; Valková, V.; Vukovic, N.L.; Vukic, M.; Terentjeva, M.; Štefániková, J.; Ďúranová, H.; Kowalczewski, P.Ł.; Kačániová, M. Thymus Serpyllum Essential Oil and Its Biological Activity as a Modern Food Preserver. *Plants* **2021**, *10*, 1416. [CrossRef] [PubMed]
9. Kizi, S.D.S. Pharmacognostic Analysis of Medicinal Plants Containing Essential Oils. *Texa. Jour. Medi. Scie.* **2023**, *25*, 99–102. [CrossRef]
10. Uritu, C.M.; Mihai, C.T.; Stanciu, G.-D.; Dodi, G.; Alexa-Stratulat, T.; Luca, A.; Leon-Constantin, M.-M.; Stefanescu, R.; Bild, V.; Melnic, S.; et al. Medicinal Plants of the Family *Lamiaceae* in Pain Therapy: A Review. *Pain. Res. Manag.* **2018**, *2018*, 7801543. [CrossRef]
11. Waller, S.B.; Cleff, M.B.; Serra, E.F.; Silva, A.L.; Gomes, A.D.R.; de Mello, J.R.B.; de Faria, R.O.; Meireles, M.C.A. Plants from *Lamiaceae* Family as Source of Antifungal Molecules in Humane and Veterinary Medicine. *Microb. Pathog.* **2017**, *104*, 232–237. [CrossRef]
12. De Martino, L.; De Feo, V.; Nazzaro, F. Chemical Composition and in Vitro Antimicrobial and Mutagenic Activities of Seven Lamiaceae Essential Oils. *Molecules* **2009**, *14*, 4213–4230. [CrossRef] [PubMed]
13. Ipek, E.; Sivas Zeytinoglu, H.; Okay, S.; Tuylu, B.; Kurkcuoglu, M.; Baser, K.H.C. Genotoxicity and Antigenotoxicity of Origanum Oil and Carvacrol Evaluated by Ames Salmonella/Microsomal Test. *Food Chem.* **2005**, *93*, 551–556. [CrossRef]
14. Evandri, M.G.; Battinelli, L.; Daniele, C.; Mastrangelo, S.; Bolle, P.; Mazzanti, G. The Antimutagenic Activity of *Lavandula angustifolia* (Lavender) Essential Oil in the Bacterial Reverse Mutation Assay. *Food Chem. Toxicol.* **2005**, *43*, 1381–1387. [CrossRef] [PubMed]

15. Vizoso Parra, A.; Ramos Ruiz, A.; Decalo Michelena, M.; Betancourt Badell, J. Estudio Genotóxico in Vitro e in Vivo En Tinturas de *Melissa officinalis* L. (Toronjil) y *Mentha piperita* L. (Toronjil de Menta). *Rev. Cuba. Plantas Med.* **1997**, *2*, 6–11.
16. Vuković-Gacić, B.; Nikcević, S.; Berić-Bjedov, T.; Knezević-Vukcević, J.; Simić, D. Antimutagenic Effect of Essential Oil of Sage (*Salvia officinalis* L.) and Its Monoterpenes against UV-Induced Mutations in Escherichia Coli and Saccharomyces Cerevisiae. *Food Chem. Toxicol.* **2006**, *44*, 1730–1738. [CrossRef]
17. Sun, J.; Sun, P.; Kang, C.; Zhang, L.; Guo, L.; Kou, Y. Chemical Composition and Biological Activities of Essential Oils from Six Lamiaceae Folk Medicinal Plants. *Front. Plant Sci.* **2022**, *13*, 919294. [CrossRef]
18. Ramos da Silva, L.R.; Ferreira, O.O.; Cruz, J.N.; de Jesus Pereira Franco, C.; Oliveira Dos Anjos, T.; Cascaes, M.M.; Almeida da Costa, W.; Helena de Aguiar Andrade, E.; Santana de Oliveira, M. *Lamiaceae* Essential Oils, Phytochemical Profile, Antioxidant, and Biological Activities. *Evid. Based Complement. Altern. Med.* **2021**, *2021*, 6748052. [CrossRef]
19. Benyoucef, F.; Dib, M.E.A.; Arrar, Z.; Costa, J.; Muselli, A. Synergistic Antioxidant Activity and Chemical Composition of Essential Oils From *Thymus fontanesii*, *Artemisia herba-alba* and *Rosmarinus officinalis*. *J. Apple Biotechnol. Rep.* **2018**, *5*, 151–156. [CrossRef]
20. Shanaida, M. Antioxidant Activity of Essential Oils Obtained from Aerial Part of Some *Lamiaceae* Species. *Int. J. Green Pharm.* **2018**, *12*, 200–204. [CrossRef]
21. Mutlu İngök, A.; Çatalkaya, G.; Çapanoğlu, E.; Karbancıoğlu Güler, F. Antioxidant and Antimicrobial Activities of Fennel, Ginger, Oregano and Thyme Essential Oils. *Food Front.* **2021**, *2*, 508–518. [CrossRef]
22. Jafari, S.; Mori, Y. Chemical Composition and Antioxidant Activity of Essential Oil of *Coriandrum sativum* L. Seeds Cultivated in Afghanistan. *Eur. J. Med. Plants* **2021**, *32*, 82–92. [CrossRef]
23. Ahmadi-Dastgerdi, A.; Ezzatpanah, H.; Asgary, S.; Dokhani, S.; Rahimi, E.; Gholami-Ahangaran, M. Oxidative Stability of Mayonnaise Supplemented with Essential Oil of Achillea Millefolium Ssp Millefolium during Storage. *Food Sci. Technol.* **2019**, *13*, 34–41. [CrossRef]
24. Ibáñez, M.D.; López-Gresa, M.P.; Lisón, P.; Rodrigo, I.; Bellés, J.M.; González-Mas, M.C.; Blázquez, M.A. Essential Oils as Natural Antimicrobial and Antioxidant Products in the Agrifood Industry. *Nereis* **2020**, *12*, 55–69. [CrossRef]
25. Tsigarida, E.; Skandamis, P.; Nychas, G.J. Behaviour of Listeria Monocytogenes and Autochthonous Flora on Meat Stored under Aerobic, Vacuum and Modified Atmosphere Packaging Conditions with or without the Presence of Oregano Essential Oil at 5 Degrees C. *J. Appl. Microbiol.* **2000**, *89*, 901–909. [CrossRef]
26. Duarte, A.E.; De Menezes, I.R.A.; Bezerra Morais Braga, M.F.; Leite, N.F.; Barros, L.M.; Waczuk, E.P.; Pessoa da Silva, M.A.; Boligon, A.; Teixeira Rocha, J.B.; Souza, D.O.; et al. Antimicrobial Activity and Modulatory Effect of Essential Oil from the Leaf of Rhaphiodon Echinus (Nees & Mart) Schauer on Some Antimicrobial Drugs. *Molecules* **2016**, *21*, 743. [CrossRef] [PubMed]
27. Karpiński, T.M. Essential Oils of Lamiaceae Family Plants as Antifungals. *Biomolecules* **2020**, *10*, 103. [CrossRef] [PubMed]
28. Kowalczyk, T.; Merecz-Sadowska, A.; Ghorbanpour, M.; Szemraj, J.; Piekarski, J.; Bijak, M.; Śliwiński, T.; Zajdel, R.; Sitarek, P. Enhanced Natural Strength: Lamiaceae Essential Oils and Nanotechnology in In Vitro and In Vivo Medical Research. *Int. J. Mol. Sci.* **2023**, *24*, 15279. [CrossRef]
29. Monzote, L.; Scherbakov, A.; Lizama, R.; Gutiérrez, Y.; Satyal, P.; Cos, P.; Shchekotikhin, A.; Gille, L. Pharmacological Assessment of the Carvacrol Chemotype Essential Oil From Plectranthus Amboinicus Growing in Cuba. *Nat. Product. Commun.* **2020**, *15*, 1934578X2096223. [CrossRef]
30. Kim, S.-W.; Lee, H.-R.; Jang, M.-J.; Jung, C.-S.; Park, I.-K. Fumigant Toxicity of Lamiaceae Plant Essential Oils and Blends of Their Constituents against Adult Rice Weevil *Sitophilus oryzae*. *Molecules* **2016**, *21*, 361. [CrossRef]
31. Wang, L.; Cao, Z.; Wang, Z.; Guo, J.; Wen, J. Reactive Oxygen Species Associated Immunoregulation Post Influenza Virus Infection. *Front. Immunol.* **2022**, *13*, 927593. [CrossRef]
32. Battaglini, M.; Carmignani, A.; Martinelli, C.; Colica, J.; Marino, A.; Doccini, S.; Mollo, V.; Santoro, F.; Bartolucci, M.; Petretto, A.; et al. In Vitro Study of Polydopamine Nanoparticles as Protective Antioxidant Agents in Fibroblasts Derived from ARSACS Patients. *Biomater. Sci.* **2022**, *10*, 3770–3792. [CrossRef] [PubMed]
33. Martinelli, C.; Pucci, C.; Battaglini, M.; Marino, A.; Ciofani, G. Antioxidants and Nanotechnology: Promises and Limits of Potentially Disruptive Approaches in the Treatment of Central Nervous System Diseases. *Adv. Healthc. Mater.* **2020**, *9*, e1901589. [CrossRef] [PubMed]
34. Klimova, N.; Fearnow, A.; Kristian, T. Role of NAD+-Modulated Mitochondrial Free Radical Generation in Mechanisms of Acute Brain Injury. *Brain Sci.* **2020**, *10*, 449. [CrossRef]
35. Perillo, B.; Di Donato, M.; Pezone, A.; Di Zazzo, E.; Giovannelli, P.; Galasso, G.; Castoria, G.; Migliaccio, A. ROS in Cancer Therapy: The Bright Side of the Moon. *Exp. Mol. Med.* **2020**, *52*, 192–203. [CrossRef] [PubMed]
36. Iliadis, S.; Papanikolaou, N.A. Reactive Oxygen Species Mechanisms That Regulate Protein–Protein Interactions in Cancer. *Int. J. Mol. Sci.* **2024**, *25*, 9255. [CrossRef]
37. Ju, S.; Singh, M.K.; Han, S.; Ranbhise, J.; Ha, J.; Choe, W.; Yoon, K.-S.; Yeo, S.G.; Kim, S.S.; Kang, I. Oxidative Stress and Cancer Therapy: Controlling Cancer Cells Using Reactive Oxygen Species. *Int. J. Mol. Sci.* **2024**, *25*, 12387. [CrossRef]

38. Wang, L.; Wise, J.T.F.; Zhang, Z.; Shi, X. Progress and Prospects of Reactive Oxygen Species in Metal Carcinogenesis. *Curr. Pharmacol. Rep.* **2016**, *2*, 178–186. [CrossRef]
39. Khokra, S.; Prakash, O.; Jain, S. A Study on Neurological Significance of Vitex Negundo Linn. *Int. Res. J. Pharm.* **2018**, *9*, 130–136. [CrossRef]
40. Rahmi, D.; Yunilawati, R.; Setiawati, I.; Irwinanita, I.; Jati, B.; Riyanto, A.; Yemirta, Y.; Aidha, N. Antioxidant Activity, Skin Irritaion Potential and Chemical Composition of Clove Leaf Oil from West Java Indonesia. *J. Sains Mater. Indones.* **2021**, *23*, 24. [CrossRef]
41. Spiridon, I.; Colceru, S.; Anghel, N.; Teaca, C.A.; Bodirlau, R.; Armatu, A. Antioxidant Capacity and Total Phenolic Contents of Oregano (*Origanum vulgare*), Lavender (*Lavandula angustifolia*) and Lemon Balm (*Melissa officinalis*) from Romania. *Nat. Prod. Res.* **2011**, *25*, 1657–1661. [CrossRef]
42. Robu, S.; Aprotosoaie, A.C.; Miron, A.; Cioanca, O.; Stănescu, U.; Hancianu, M. In Vitro Antioxidant Activity of Ethanolic Extracts from Some *Lavandula* Species Cultivated in Romania. *Farmacia* **2012**, *60*, 394–401.
43. Massoud, R.I.; Bouaziz, M.; Abdallah, H.; Zeiz, A.; Flamini, G.; El-Dakdouki, M.H. Comparative Study on the Chemical Composition and Biological Activities of the Essential Oils of *Lavandula angustifolia* and *Lavandula* × *intermedia* Cultivated in Lebanon. *ACS Omega* **2024**, *9*, 30244–30255. [CrossRef] [PubMed]
44. Truzzi, E.; Chaouch, M.A.; Rossi, G.; Tagliazucchi, L.; Bertelli, D.; Benvenuti, S. Characterization and Valorization of the Agricultural Waste Obtained from Lavandula Steam Distillation for Its Reuse in the Food and Pharmaceutical Fields. *Molecules* **2022**, *27*, 1613. [CrossRef]
45. Hasibi, A.; Abdossi, V.; Ladanmoghadam, A.; Moradi, P. Variation of Some Traits of *Lavandula angustifolia* to Drought Stress for Optimum Water Usage. *Eur. J. Hortic. Sci.* **2022**, *87*, 1–8. [CrossRef] [PubMed]
46. Bogdan, M.; Bungau, S.; Tit, D.M.; Copolovici, L.; Behl, T.; Otrisal, P.; Aleya, L.; Cioca, G.; Berescu, D.; Uivarosan, D.; et al. Variations in the Chemical Composition of the Essential Oil of *Lavandula angustifolia* Mill., Moldoveanca 4 Romanian Variety. *Rev. Chim.* **2020**, *71*, 307–315. [CrossRef]
47. Kamali, H.; Jalilvand, M.R.; Aminimoghadamfarouj, N. Pressurized Fluid Extraction of Essential Oil from Lavandula Hybrida Using a Modified Supercritical Fluid Extractor and a Central Composite Design for Optimization. *J. Sep. Sci.* **2012**, *35*, 1479–1485. [CrossRef]
48. Messaoud, C.; Chograni, H.; Boussaid, M. Chemical Composition and Antioxidant Activities of Essential Oils and Methanol Extracts of Three Wild *Lavandula* L. Species. *Nat. Prod. Res.* **2012**, *26*, 1976–1984. [CrossRef]
49. Ghanimi, R.; Ouhammou, A.; Atki, Y.E.; Cherkaoui, M. Antioxidant and Antibacterial Activities of Essential Oils from Three Moroccan Species (*Lavandula mairei* Humbert, *Lavandula dentata* L. and, *Lavandula stoechas* L.). *J. Pharm. Res. Int.* **2021**, *33*, 64–71. [CrossRef]
50. Moharam, B.A.; Jantan, I.; Ahmad, F.B.; Jalil, J. Antiplatelet Aggregation and Platelet Activating Factor (PAF) Receptor Antagonistic Activities of the Essential Oils of Five *Goniothalamus* Species. *Molecules* **2010**, *15*, 5124–5138. [CrossRef]
51. Eldeghedy, H.I.; El-Gendy, A.E.-N.G.; Nassrallah, A.A.; Aboul-Enein, A.M.; Omer, E.A. Chemical Composition of Essential Oils of *Lavandula angustifolia* and Lavandula Hybrida Cultivated in Egypt and Their Biological Activities. *Egypt. J. Chem.* **2022**, *65*, 595–610. [CrossRef]
52. Afonso, A.F.; Pereira, O.R.; Fernandes, Â.; Calhelha, R.C.; Silva, A.M.S.; Ferreira, I.C.F.R.; Cardoso, S.M. Phytochemical Composition and Bioactive Effects of Salvia Africana, *Salvia officinalis* 'Icterina' and Salvia Mexicana Aqueous Extracts. *Molecules* **2019**, *24*, 4327. [CrossRef]
53. Jažo, Z.; Glumac, M.; Paštar, V.; Bektić, S.; Radan, M.; Carev, I. Chemical Composition and Biological Activity of *Salvia officinalis* L. Essential Oil. *Plants* **2023**, *12*, 1794. [CrossRef] [PubMed]
54. El Jery, A.; Hasan, M.; Rashid, M.M.; Al Mesfer, M.K.; Danish, M.; Ben Rebah, F. Phytochemical Characterization, and Antioxidant and Antimicrobial Activities of Essential Oil from Leaves of the Common Sage *Salvia officinalis* L. from Abha, Saudi Arabia. *Asian Biomed. (Res. Rev. News)* **2020**, *14*, 261–270. [CrossRef]
55. Robu, S.; Romila, A.; Buzia, O.D.; Spac, A.F.; Diaconu, C.; Tutunaru, D.; Lisa, E.; Nechita, A. Contribution to the Optimization of a Gas Chromatographic Method by QbD Approach Used for Analysis of Essential Oils from *Salvia officinalis*. *Rev. Chim.* **2019**, *70*, 2015–2020. [CrossRef]
56. Angelova, V. Heavy Metal Accumulation and Chemical Composition of Essential Oils of *Salvia officinalis* Cultivated on Heavy Metal Contaminated Soils. 2019; 54. [CrossRef]
57. Tsai, S.-W.; Hsieh, M.-C.; Li, S.; Lin, S.-C.; Wang, S.-P.; Lehman, C.W.; Lien, C.Z.; Lin, C.-C. Therapeutic Potential of Sclareol in Experimental Models of Rheumatoid Arthritis. *Int. J. Mol. Sci.* **2018**, *19*, 1351. [CrossRef] [PubMed]
58. Gad, H.A.; Mamadalieva, R.Z.; Khalil, N.; Zengin, G.; Najar, B.; Khojimatov, O.K.; Al Musayeib, N.M.; Ashour, M.L.; Mamadalieva, N.Z. GC-MS Chemical Profiling, Biological Investigation of Three *Salvia* Species Growing in Uzbekistan. *Molecules* **2022**, *27*, 5365. [CrossRef] [PubMed]

59. Kačániová, M.; Vukovic, N.L.; Čmiková, N.; Galovičová, L.; Schwarzová, M.; Šimora, V.; Kowalczewski, P.Ł.; Kluz, M.I.; Puchalski, C.; Bakay, L.; et al. *Salvia sclarea* Essential Oil Chemical Composition and Biological Activities. *Int. J. Mol. Sci.* **2023**, *24*, 5179. [CrossRef]
60. Ben Akacha, B.; Ben Hsouna, A.; Generalić Mekinić, I.; Ben Belgacem, A.; Ben Saad, R.; Mnif, W.; Kačániová, M.; Garzoli, S. *Salvia officinalis* L. and *Salvia sclarea* Essential Oils: Chemical Composition, Biological Activities and Preservative Effects against Listeria Monocytogenes Inoculated into Minced Beef Meat. *Plants* **2023**, *12*, 3385. [CrossRef]
61. Aćimović, M.G.; Cvetković, M.T.; Stanković Jeremić, J.M.; Pezo, L.L.; Varga, A.O.; Čabarkapa, I.S.; Kiprovski, B. Biological Activity and Profiling of *Salvia sclarea* Essential Oil Obtained by Steam and Hydrodistillation Extraction Methods via Chemometrics Tools. *Flavour. Fragr. J.* **2022**, *37*, 20–32. [CrossRef]
62. Salehi, B.; Stojanović-Radić, Z.; Matejić, J.; Sharopov, F.; Antolak, H.; Kręgiel, D.; Sen, S.; Sharifi-Rad, M.; Acharya, K.; Sharifi-Rad, R.; et al. Plants of Genus Mentha: From Farm to Food Factory. *Plants* **2018**, *7*, 70. [CrossRef]
63. Singh, P.; Pandey, A.K. Prospective of Essential Oils of the Genus *Mentha* as Biopesticides: A Review. *Front. Plant Sci.* **2018**, *9*, 1295. [CrossRef] [PubMed]
64. Ahmed, H.; Ouis, N.; Djilali, B.; Benatouche, Z. In Vitro Antioxidant Activity of Essential Oil of Aerial Parts of *Mentha pulegium* L. *Acta Agric. Serbica* **2020**, *25*, 193–197. [CrossRef]
65. Mondher, B. Comparative Study of the Chemical Properties and Composition of the *Mentha pulegium* L. of Algerian Origin and That of Tunisian Origin. *Int. J. Agric. Sci. Food Technol.* **2020**, *6*, 030–036. [CrossRef]
66. Gonçalves, R.S.; Battistin, A.; Pauletti, G.; Rota, L.; Serafini, L.A. Antioxidant Properties of Essential Oils from *Mentha* Species Evidenced by Electrochemical Methods. *Rev. Bras. Plantas Med.* **2009**, *11*, 372–382. [CrossRef]
67. Brahmi, F.; Khodir, M.; Mohamed, C.; Pierre, D.; Brahmi, F.; Khodir, M.; Mohamed, C.; Pierre, D. Chemical Composition and Biological Activities of *Mentha* Species. In *Aromatic and Medicinal Plants—Back to Nature*; IntechOpen: Rijeka, Croatia, 2017. [CrossRef]
68. Singh, R.; Shushni, M.; Belkheir, A. Antibacterial and Antioxidant Activities of *Mentha piperita* L. *Arab. J. Chem.* **2015**, *8*, 322–328. [CrossRef]
69. Mimica-Dukić, N.; Bozin, B.; Soković, M.; Mihajlović, B.; Matavulj, M. Antimicrobial and Antioxidant Activities of Three *Mentha* Species Essential Oils. *Planta Med.* **2003**, *69*, 413–419. [CrossRef]
70. Kianasab, M.R.; Mohammadhosseini, M.; Nekoei, M.; Mahdavi, B.; Baheri, T. Screening of the Compositions of Essential Oils and Volatiles of Perovskia Abrotanoides Karel. along with Antioxidant, Antibacterial and Cytotoxic Impacts of Its Methanol Extract. *Nat. Prod. Res.* **2024**, *38*, 3813–3817. [CrossRef]
71. Ghaffari, Z.; Rahimmalek, M.; Sabzalian, M.R. Variations in Essential Oil Composition and Antioxidant Activity in Perovskia Abrotanoides Kar. Collected from Different Regions in Iran. *Chem. Biodivers.* **2018**, *15*, e1700565. [CrossRef]
72. Soumahoro, B.; Bohui, G.S.-P.; Kalo, M.; Kanaté, L.; Attioua, B.; Soro, Y. Compound Identification by HPLC-ESI-Q-TOF-MS/ MS Analysis of the Dichloromethane Fraction of *Hyptis suaveolens* Leaves After Extraction of the Essential Oil. *Sci. J. Chem.* **2024**, *12*, 1–14. [CrossRef]
73. Bajalan, I.; Rouzbahani, R.; Pirbalouti, A.G.; Maggi, F. Chemical Composition and Antibacterial Activity of Iranian *Lavandula* × *hybrida*. *Chem. Biodivers.* **2017**, *14*, e1700064. [CrossRef]
74. de Rapper, S.; Viljoen, A.; van Vuuren, S. The In Vitro Antimicrobial Effects of *Lavandula angustifolia* Essential Oil in Combination with Conventional Antimicrobial Agents. *Evid. Based Complement. Altern. Med.* **2016**, *2016*, 2752739. [CrossRef] [PubMed]
75. Karadağ, A.E.; İpekçi, E.; Yağcılar, A.P.; Demirbolat, İ.; Kartal, M.; Siafaka, P.I.; Okur, N.Ü. Antimicrobial Activities of Mouthwashes Obtained from Various Combinations of *Elettaria cardamomum* Maton., *Lavandula angustifolia* Mill. and *Salvia triloba* L. Essential Oils. *Nat. Volatiles Essent. Oils* **2020**, *7*, 9–17. [CrossRef]
76. Adaszyńska-Skwirzyńska, M.; Szczerbińska, D. The Antimicrobial Activity of Lavender Essential Oil (*Lavandula angustifolia*) and Its Influence on the Production Performance of Broiler Chickens. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 1020–1025. [CrossRef]
77. Biltekin, S.N.; Karadağ, A.E.; Demirci, B.; Demirci, F. ACE2 and LOX Enzyme Inhibitions of Different Lavender Essential Oils and Major Components Linalool and Camphor. *ACS Omega* **2022**, *7*, 36561–36566. [CrossRef]
78. Tofah, M.L.; Mseddi, K.; Al-Abbasi, O.K.; Ben Yazid, A.; Khechine, A.; Gdoura, R.; Khannous, L. A New Lavender (*Lavandula multifida* L.) Ecotype from Arid Tunisia, with Differential Essential Oil Composition and Higher Antimicrobial Potential. *Life* **2023**, *13*, 103. [CrossRef]
79. Álvarez-García, S.; Moumni, M.; Romanazzi, G. Antifungal Activity of Volatile Organic Compounds from Essential Oils against the Postharvest Pathogens Botrytis Cinerea, *Monilinia fructicola*, *Monilinia fructigena*, and *Monilinia laxa*. *Front. Plant Sci.* **2023**, *14*, 1274770. [CrossRef]
80. Bozkurt, İ.A.; Soylu, S.; Kara, M.; Soylu, E.M. Chemical Composition and Antibacterial Activity of Essential Oils Isolated from Medicinal Plants against Gall Forming Plant Pathogenic Bacterial Disease Agents. *KSU J. Agric. Nat.* **2020**, *23*, 1474–1482. [CrossRef]

81. Derradji, L.; Saidi, O.; Hadef, Y. Evaluation of the Antibacterial Activity of Three Essential Oils Extracted from Plants Used in Traditional Medicine in Algeria (*Salvia officinalis* L, *Melissa officinalis* L and *Origanum vulgare* L). *GSC Biol. Pharm. Sci.* **2020**, *12*, 181–188. [CrossRef]
82. Alibi, S.; Asma, F.; Ben Mansour, H.; Navas, J. In Vitro Antibacterial Effects of *Salvia sclarea*, Eucalyptus Globulus and Eugenia Caryophyllata Essential Oils Against Multidrug Resistant Corynebacterium Spp Clinical Isolates. *J. Clin. Res. Rep.* **2020**, *2*, 1–5. [CrossRef]
83. Ürgeová, E.; Uváčková, Ľ.; Vaneková, M.; Maliar, T. Antibacterial Potential of Microwave-Assisted Extraction Prepared Hydrolates from Different *Salvia* Species. *Plants* **2023**, *12*, 1325. [CrossRef]
84. Küçük, S.; Soyer, P.; Tunali, Y. Determination of Antimicrobial and Biological Activities of *Salvia sclarea* L. (Lamiaceae) Extracts. *J. Turk. Chem. Soc. Sect. A Chem.* **2019**, *6*, 15–20. [CrossRef]
85. Akotowanou, O.C.A.; Adjou, E.S.; Sessou, P.; Kougblenou, S.D.; Olubi, A.B.; Michels, F.; Ahoussi, E.D.; Yessoufou, A.; Bankolé, H.; Fauconnier, M.-L.; et al. Antifungal Properties of *Pimenta racemosa* (Mill.) and *Mentha* × *piperita* (L.) Essential Oils against Fusarium Oxysporum Causing Tomato Fruit Rot. *J. Adv. Biol. Biotechnol.* **2023**, *26*, 50–59. [CrossRef]
86. Giridharan, B.; Amutha, C.; Siddhan, N.; Ganeshkumar, A.; Periyasamy, S.; Murali, K. Antibacterial Activity of *Mentha piperita* L. (Peppermint) from Leaf Extracts—A Medicinal Plant. *Acta Agric. Slov.* **2007**, *89*, 73–79. [CrossRef]
87. Jianu, C.; Golet, I.; Misca, C.; Jianu, A.; Pop, G.; Lukinich-Gruia, A. Antimicrobial Properties and Chemical Composition of Essential Oils Isolated from Six Medicinal Plants Grown in Romania Against Foodborne Pathogens. *Rev. Chim.-Buchar.—Orig. Ed.* **2016**, *67*, 1056–1061.
88. Hamad Al-Mijalli, S.; ELsharkawy, E.R.; Abdallah, E.M.; Hamed, M.; El Omari, N.; Mahmud, S.; Alshahrani, M.M.; Mrabti, H.N.; Bouyahya, A. Determination of Volatile Compounds of *Mentha piperita* and *Lavandula multifida* and Investigation of Their Antibacterial, Antioxidant, and Antidiabetic Properties. *Evid. Based Complement. Altern. Med.* **2022**, *2022*, 9306251. [CrossRef]
89. Demir, H. Evaluation of the Chemical Profiling, Total Phenolic Composition, the Antioxidant and Antimicrobial Properties of the Essential Oils of *Mentha piperita* L., *Salvia officinalis* L., and *Thymus vulgaris* L. *J. Food Sci. Eng.* **2018**, *8*, 263–270. [CrossRef]
90. Haydari, M.; Maresca, V.; Rigano, D.; Taleei, A.; Shahnejat-Bushehri, A.A.; Hadian, J.; Sorbo, S.; Guida, M.; Manna, C.; Piscopo, M.; et al. Salicylic Acid and Melatonin Alleviate the Effects of Heat Stress on Essential Oil Composition and Antioxidant Enzyme Activity in *Mentha* × *piperita* and *Mentha arvensis* L. *Antioxidants* **2019**, *8*, 547. [CrossRef]
91. Vakili-Ghartavol, M.; Arouiee, H.; Golmohammadzadeh, S.; Naseri, M. Antifungal Activity of *Mentha* × *piperita* L. Essential Oil. *Acta Sci. Pol. Hortorum Cultus* **2022**, *21*, 143–152. [CrossRef]
92. Tullio, V.; Roana, J.; Scalas, D.; Mandras, N. Evaluation of the Antifungal Activity of *Mentha* × *piperita* (Lamiaceae) of Pancalieri (Turin, Italy) Essential Oil and Its Synergistic Interaction with Azoles. *Molecules* **2019**, *24*, 3148. [CrossRef]
93. Edris, A.; Jirovetz, L.; Buchbauer, G.; Denkova, Z.; Stoyanova, A.; Slavchev, A. Chemical Composition, Antimicrobial Activities and Olfactive Evaluation of a *Salvia officinalis* L. (Sage) Essential Oil from Egypt. *J. Essent. Oil Res.* **2007**, *19*, 186–189. [CrossRef]
94. Camargo, K.C.; Batista, L.R.; Alves, E.; Rezende, D.A.d.C.S.; Teixeira, M.L.; Brandão, R.M.; Ferreira, V.R.F.; Nelson, D.L.; Cardoso, M.d.G. Antibacterial Action of the Essential Oil from Cantinoa Carpinifolia Benth. Against Escherichia Coli and Staphylococcus Aureus Strains. *Flavour. Fragr. J.* **2020**, *35*, 99–106. [CrossRef]
95. Nath, S.; Tamuli, K.J.; Saikia, S.; Narzary, B.; Gogoi, B.; Bordoloi, M.; Neipihoi; Dutta, D.; Sahoo, R.K.; Das, A.; et al. Essential Oil from the Leaves of Elsholtzia Communis (Collett & Hemsl.) Diels from North East India: Studies on Chemical Profiling, Antimicrobial, Cytotoxic and ACE Inhibitory Activities. *Flavour. Fragr. J.* **2021**, *36*, 626–636. [CrossRef]
96. Alexa, E.; Danciu, C.; Radulov, I.; Obistioiu, D.; Sumalan, R.M.; Morar, A.; Dehelean, C.A. Phytochemical Screening and Biological Activity of *Mentha* × *piperita* L. and *Lavandula angustifolia* Mill. Extracts. *Anal. Cell. Pathol.* **2018**, *2018*, 2678924. [CrossRef]
97. Cocan, I.; Alexa, E.; Danciu, C.; Radulov, I.; Galuscan, A.; Obistioiu, D.; Morvay, A.A.; Sumalan, R.M.; Poiana, M.-A.; Pop, G.; et al. Phytochemical Screening and Biological Activity of Lamiaceae Family Plant Extracts. *Exp. Ther. Med.* **2017**, *15*, 1863. [CrossRef] [PubMed]
98. Alexa, E.; Sumalan, R.M.; Danciu, C.; Obistioiu, D.; Negrea, M.; Poiana, M.-A.; Rus, C.; Radulov, I.; Pop, G.; Dehelean, C. Synergistic Antifungal, Allelopatic and Anti-Proliferative Potential of *Salvia officinalis* L., and *Thymus vulgaris* L. Essential Oils. *Molecules* **2018**, *23*, 185. [CrossRef]
99. NIST 14 Mass Spec Library and Search Programs—User Manual. Available online: https://www.sisweb.com/manuals/nist.htm (accessed on 24 February 2025).
100. Wiley GCMS Libraries. MS Wil. Available online: https://www.mswil.com/software/spectral-libraries-and-databases/wiley-spectral-libraries/wiley-gcms-libraries/ (accessed on 24 February 2025).
101. Kedare, S.B.; Singh, R.P. Genesis and Development of DPPH Method of Antioxidant Assay. *J. Food Sci. Technol.* **2011**, *48*, 412–422. [CrossRef]
102. Moacă, E.-A.; Pavel, I.Z.; Danciu, C.; Crăiniceanu, Z.; Minda, D.; Ardelean, F.; Antal, D.S.; Ghiulai, R.; Cioca, A.; Derban, M.; et al. Romanian Wormwood (*Artemisia absinthium* L.): Physicochemical and Nutraceutical Screening. *Molecules* **2019**, *24*, 3087. [CrossRef]

103. Niu, Y.; Yang, C.; Zhou, J.; Huang, S.; Liu, J. Two New Compounds with Antimicrobial Activities from the Seeds of *Voacanga africana*. *Phytochem. Lett.* **2016**, *18*, 208–212. [CrossRef]
104. Clinical & Laboratory Standards Institute: CLSI Guidelines. Clinical & Laboratory Standards Institute. Available online: https://clsi.org/ (accessed on 19 October 2024).
105. M26 AE Bactericidal Activity of Antimicrobial Agents. Clinical & Laboratory Standards Institute. Available online: https://clsi.org/standards/products/microbiology/documents/m26/ (accessed on 9 December 2024).
106. Bobadilla, A.V.P.; Arévalo, J.; Sarró, E.; Byrne, H.M.; Maini, P.K.; Carraro, T.; Balocco, S.; Meseguer, A.; Alarcón, T. In Vitro Cell Migration Quantification Method for Scratch Assays. *J. R. Soc. Interface* **2019**, *16*, 20180709. [CrossRef]
107. Pusnik, M.; Imeri, M.; Deppierraz, G.; Bruinink, A.; Zinn, M. The Agar Diffusion Scratch Assay—A Novel Method to Assess the Bioactive and Cytotoxic Potential of New Materials and Compounds. *Sci. Rep.* **2016**, *6*, 20854. [CrossRef]
108. MTT Assay Protocol | Abcam. Available online: https://www.abcam.com/en-us/technical-resources/protocols/mtt-assay?srsltid=AfmBOorh9g61ejVubQ9usSO9HBZVEuIeQBu5txXHKARmWO02oMVNeccU (accessed on 9 December 2024).
109. Coricovac, D.-E.; Moacă, E.-A.; Pinzaru, I.; Cîtu, C.; Soica, C.; Mihali, C.-V.; Păcurariu, C.; Tutelyan, V.A.; Tsatsakis, A.; Dehelean, C.-A. Biocompatible Colloidal Suspensions Based on Magnetic Iron Oxide Nanoparticles: Synthesis, Characterization and Toxicological Profile. *Front. Pharmacol.* **2017**, *8*, 154. [CrossRef] [PubMed]
110. PAST. LO4D.com. Available online: https://past.en.lo4d.com/windows (accessed on 9 December 2024).
111. SAS OnDemand for Academics | SAS. Available online: https://www.sas.com/en_us/software/on-demand-for-academics.html (accessed on 9 December 2024).
112. Prism 5 Updates—GraphPad. Available online: https://www.graphpad.com/support/prism-5-updates/ (accessed on 9 December 2024).

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Cornus mas: From Plant Taxonomy and Distribution Area to Highly Valorization of Phytochemicals by Microencapsulation in Biopolymeric Matrices Containing Probiotics

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Abstract

In this study, a comprehensive approach to the taxonomy and the distribution areas of *Cornus mas* (commonly known as cornelian cherry) is presented, considering the superior valorization of bioactive compounds through co-microencapsulation in a unique matrix combination, together with probiotic bacteria. According to the phytochemical profile, the whole plant of cornelian cherry includes 101 chemical compounds, classified as follows: polyphenols, terpenoids, carotenoids, vitamins, carbohydrates, acids, and hydrocarbons. In general, the bioactive compounds are highly sensitive to digestion and external factors, such as oxygen, pH, temperature, etc. In order to improve the bioaccesibility and the storage stability of the polyphenols, a solid–liquid ultrasound assisted method was applied to deliver an anthocyanin-enriched extract, which was microencapsulated together with *Lacticaseibacillus casei (L. casei)* by freeze-drying in a unique combination of whey protein isolate (WPI) and maltodextrin (MD) as wall materials. Two powders were obtained, with and without the probiotic bacteria. The data obtained in this study showed a high encapsulation efficiency (82.16–88.95%) of anthocyanins, whereas for *L. casei*, the microencapsulation efficiency reached 80%. The co-microencapsulated powder showed a viable cell count of $3.80 \cdot 10^9$ CFU/g dry matter (D.M.). The microencapsulated powders showed a significant amount of total polyphenols (8.30–13.00 mg of gallic acid equivalent per gram D.M.). Furthermore, the in vitro digestibility of the anthocyanins highlighted the protective effect of the microencapsulation matrix in the stomach, whereas a slow release was observed in the simulated intestinal conditions. Furthermore, after 21 days of storage, the lactic acid bacteria viability was high (2.53×10^9 CFU/g dry matter), which confirmed the functionality and the nutraceutical value of the co-microencapsulated powder.

Keywords: anthocyanins; microencapsulation; *Cornus mas*; maltodextrin; whey protein isolate; *Lacticaseibacillus casei*

1. Introduction

Most of the current research has focused on the beneficial role of plant phytochemicals for human health, including their antioxidant and anti-inflammatory capacities, their antidiabetic and anticancer activities, and their effects on preventing neuronal illnesses or reducing cardiovascular diseases. In this context, numerous studies have highlighted the significant impact of anthocyanins on human health and the advantages provided as naturally occurring, plant-derived, red, violet, and blue pigments [1–3]. Additionally, food's color is one of the most significant organoleptic qualities, indicating both the freshness of foods and its anthocyanin content. The natural anthocyanins have recently received more attention due to the opportunities to replace the synthetic dies in the food industry and as nutraceuticals.

Cornus mas L. is one of most important edible forest fruits found in spontaneous crops in Romania (38 areas) [4]. The plant's bioactive compounds are recognized for their important beneficial effects on asthma, rheumatism, cardiovascular disease, anemia, haemorrhoids, cancer, inflammation, stomach ulcers, colitis, and kidney disorders. Different reports showed that the biologically active components (BACs) found in cornelian cherry fruit are the basis for all of its ethnomedicinal benefits [5–7].

However, in order to translate the plant's bioactivity in foods and nutraceuticals to targeted functions, ongoing concerns were expressed regarding the influence of light, oxygen, storage duration, temperature, pH, etc., on the anthocyanins' stabilities [8]. Improvements in the functional properties and the protection of the anthocyanins may be achieved by extraction and microencapsulation using different techniques.

The objectives of this work were to establish a comprehensive image of the *Cornus mas* L. potential to be exploited as a source for functional foods and nutraceuticals. Therefore, in the first part of this work, important information is provided regarding the plant composition, its taxonomy, and its cultivation areas in Romania, followed by a proposed strategy to valorize the phytochemicallys enriched extract into freeze-dried powders containing polyphenolic compounds and probiotics microencapsulated in a complex matrix formed by proteins and carbohydrates. Therefore, the wall materials used in this study were chosen based on the different reports in the literature regarding the protective effects on anthocyanins and probiotics. Hence, whey protein isolate (WPI) and maltodextrin (MD) were combined in a certain ratio to obtain a self-stable matrix for the bioactive compounds and the probiotics. WPI is successfully used in the food industry due to its high protein content (over 90%). These high-quality proteins have the ability to supplement the product with nutrients that are beneficial to the human body [9]. Maltodextrin is a polymer with proven efficacy in microencapsulating phenolic compounds due to its appropriate viscosity, affordability, sweet flavor, and practical particle size [10]. *L. casei* belongs to the *Lacticaseibacillus* genus and is used in the food industry for its benefits to human health. The probiotic effects of *Lacticaseibacillus* include improving lactose digestion, reducing its intolerance symptoms [11]. The phytochemical extraction was performed by a solid–liquid ultrasound assisted extraction method, using ethanol as the solvent, followed by microencapsulation in two variants, described as follows: microencapsulation of the extract in a biopolymeric matrix containing WPI and MD (variant 1) and a co-microencapsulated variant containing WPI, MD, and *L. casei* 431® (LC) (variant 2) by freeze-drying. The powders were tested for total polyphenolic content, cell viability, in vitro digestion, and color and storage stability.

The main aim of this approach is to give a holistic view of the cornelian cherry industrial potential, from the plant to foods and nutraceuticals.

2. Results

2.1. Taxonomic Classification and Worldwide Distribution of Cornus mas

Cornelian cherry is a woodland fruit belonging to the kingdom *Plantae*, the infra-kingdom *Viridiplantae*, the super-kingdom *Streptophyta*, the sub-kingdom *Embryophyta*, the class *Magnoli*-opsyda, the super-order *Asteranae*, the order *Cornales*, the family *Cornaceae*, the genus *Cornus* L., and the species *Cornus mas* [12,13]. Over time, 28 horticultural forms and variants of *Cornus mas* L. have been discovered. They are as follows: *Cornus mas* f. *alba (Weston)* Rehder; *Cornus mas* var. *albocarpa* (C.K. Schneid.) Bean; *Cornus mas* f. *andrzejowskii* Wierdak; *Cornus mas* f. *aurea* C.K. Schneid; *Cornus mas* f./var. *aurea-elegantissima* (T. Moore), Schelle, Carriere; *Cornus mas* f. *conica* Jovan; *Cornus mas* f. *elegantissima* G. Nicholson; *Cornus erythrocarpa* St.-L; *Cornus mas* f./var. *flava infra* subsp. Publ (Weston) Rehder, Steud; *Cornus homerica* Bubani; *Cornus mas* f. *luteocarpa* (Wangerin), C.K. Schneid; *Cornus mas* f./var. *macrocarpa* (Dippel) Schelle; *Cornus mas* f. *microcarpa* Sanadze; *Cornus mas* f./var. *nana* (Carriere) C.K. Schneid; *Cornus mas* var. *oblongifolia* Jovan; *Cornus mas* f. *oxycarpa* Jovan; *Cornus mas* f. *polonica* Wierdak; *Cornus mas* f. *pyriformis* Sanadze; *Cornus mas* var. *sphaerocarpa* Cretz; *Cornus mas* f./var. *variegate* (Loudon) G. Nicholson; *Cornus mas* var. *xanthocarpa* Bean; *Cornus mascula* L.; *Cornus mascula* var. *aurea-elegantissima* T. Moore; *Cornus nudiflora* Dumort; *Cornus praecox* Stokes; *Cornus vernalis* Salisb; *Eukrania mascula* (L.) Merr.; *Macrocrpium mas* (L.) Nakai [14].

In terms of geographical distribution, *Cornus mas* L. is cultivated in Albania, Armenia, Austria, Azerbaijan, Belgium, Bosnia and Herzegovina, Bulgaria, Czech Republic, Croatia, France, Germany, Georgia, Greece, Northern Iraq, Iran, Italy, Lebanon, Luxembourg, the Balkan Peninsula, Poland, Romania, Russia, Serbia and Montenegro, Hungary, Slovakia, Slovenia, Switzerland, Syria, Ukraine, and Turkey [15], but is also used in countries such as China, Kosovo, and the United States of America [16,17]. The genus *Cornus* includes shrub species widely distributed in southern and central Europe, southwest Asia, America (north, east, south, west, and central), and eastern Africa [18]. Two species of this genus are endemic to South America and one species to tropical Africa [16,17]. Regarding the worldwide area cultivation of cornelian cherry (in ha), Georgia occupies 0.3% of the total fruit orchards (135 ha) [19], while Turkey has 1.6 million cornelian cherry trees producing 17,000 tons of fruit annually. In addition, in this country, 97% of cornelian cherry fruit production is harvested from wild flora [20]. Meanwhile, in Romania, *Cornus mas* (CM) is found in 38 areas and can be considered one of the most important forest fruits in our country. It is well known that the altitude, temperature, average annual precipitation, relief, rock type, soil type, phytocenosis structure, and floristic composition of the habitat can influence *C. mas* distribution in our country [4,8]. A native from the Caucasus [21,22], CM is one of four species that are harvested for their fruits (*C. mas*, *C. officinalis*, *C. controversa*, and *C. kousa*) and not only for ornamental purposes, as are the other species of *Cornus* [23,24]. Regarding the number of species in the *Cornaceae* family, different reports are available, such as the following: 40 species [25], 55 species [26,27], 60 species [28], and 65 species (15 red-line species and 50 blue-line species) [5,18,24].

In Figure 1, some suggestive segments of the CM plant (bark, shoots, leaves, fruits, stone, and seeds) are shown.

A study by Dinda et al. [5] provides an overview of the phytochemical profile of the whole plant of *Cornus mas*. Briefly, the cornelian cherry contains 101 phytochemical compounds, classified into anthocyanins (10), catechins (4), flavonoids (20), phenolic acids and tannins (11), monoterpenoids (9), triterpenoids (1), iridoids (5), carotenoids (10), vitamins (4), carbohydrates (3), organic acids (8), fatty acids (6), and hydrocarbons (10) (Table 1).



(**a**) bark (**b**) shoots (**c**) leaves (**d**) unripe fruit(**e**) ripe fruit(**f**) sections of ripe fruit (**g**) stones (**h**) seeds

Figure 1. *Cornus mas* plant (original photos).

Table 1. Phytochemical profile of *Cornus mas* [5,29].

No.	Chemical Category	Phytochemical Compounds	Plant Organ
1.	Polyphenolic structure compounds		
1.1	Anthocyanins	cyanidin 3-0 galactoside (1), cyanidin-3-O-glucoside (2), cyanidin 3-O-rutinoside (3), cyanidin 3-O-robinobioside (4), delphinidin-3-O-galactoside (5), pelargonidin3-O-galactoside (6), pelargonidin-3-O-glucoside (7), pelargonidin3-O-rutinoziside (8), peonidin3-O-glucoside (9), pelargonidin 3-O robinobiozid (pelargonidin 3-*O*-rhamnosylgalactoside) (10)	fruits
1.2.	Catechins	(+) catechin (11), (-) epicatechin (12), procyanidin B1 (13), procyanidin B2 (14)	fruits, leaves
1.3.	Flavonoids	kampferol-3-O-galactoside (15), kaempferol 3-O glucoside (16), kaempferol 3-O-glucuronide (17), isorhamnetin 7-O-ramnozide (18), myricetin (19), 3-O-methyl ester naringenin (20), quercetin (21), quercetin 3-O-xyloside (22), quercetin 3-O-ramnoside (23), quercetin 3-O-glucoside (24), quercetin 3-O-galactoside (25), quercetin 3-0-, trans-aromadendrin (26), quercetin 3-O-robinobioside (27), quercetin 3-O-rutinoside (rutin) (28), quercetin 3-O-glucuronide (29), quercetin 3-O-galactozil 7-O-ramnoside (30), trans aromadendrin (31), 7-acetoxy-5,2′,4′,6′-β-pentahydroxy-3-methoxychalcone (32), 7-O-aromadendrin glucoside (33), 7,3′dihydroxy-5,4′dimethoxyflufonone (34)	flowers, leaves, fruits
1.4.	Phenolic acids and tannins	quinic acid (35), chlorogenic acid (36), ellagic acid (37), ferulic acid (38), gallic acid (39), salicylic acid (40), shikimic acid (41), vanillic acid (42), p-coumaric acid (43), 3-O-cafenoylquinic acid (44), 5-O cafenoyl quinic acid (45)	flowers, fruits
2.	Terpenoids		
2.1.	Monoterpenoids	a-terpeneol (46), borneol (47), camphor (48), carvone (49), carvacrol (50), limonen (51), verbenone (52), β-tujon (53), 1,8-cineole (54)	flowers
2.2.	Triterpenoids	ursolic acid (55)	leaves
2.3.	Iridoids	loganic acid (56), cornusid (57), loganin (58), sverosid (59), secologanin (60)	fruits
3.	Carotenoids	(all-E)-neoxanthin (61), β-carotene-5,6-monooxide (62), β-carotene (63), β-cryptoxanthin (64), lutein (65), lutein-5,6-epoxide (66), luteoxanthin (67), (9Z,9′Z)-lutein (68), (9′Z)-neoxanthine (69), (13Z,13′Z)-lutein (70)	fruits
4.	Vitamins	ascorbic acid (71), α-tocopherol (72), biotin (73), riboflavine (74)	fruits
5.	Carbohydrates	glucose (75), sucrose (76), fructose (77)	fruits
6.	Acids		
6.1.	Fatty acids	linoleic acid (78), oleic acid (79), a-linolenic acid (80), palmitoleic acid (81), palmitic acid (82), 2,4-heptadienoic acid (83)	fruits, leaves
6.2.	Organic acids	citric acid (84), fumaric acid (85), isocitric acid (86), maleic acid (87), malonic acid (88), oxalic acid (89), succinic acid (90), tartaric acid (91)	fruits, leaves
7.	Hydrocarbons	decane (92), undecane (93), dodecane (94), pentadecane (95), hexadecane (96), heptadecane (97), heneicosane (98), tricosane (99), pentacosane (100), heptacosane (101)	flowers

In addition, Table 2 shows the composition of proteins, amino acids, fatty acids, macroelements, and microelements found in cornelian cherry fruit pulp.

Table 2. Protein, amino acid, fatty acid, and macro- and microelement content of *Cornus mas* [30].

Crt. No.	Identified Compound	Results	Unit of Measurement
A.	**Proteins and amino acids**		
1.	Protein	3.40	g/kg
2.	Aspartic acid	0.30	g/kg
3.	Glutamic acid	0.30	g/kg
4.	Arginine	0.30	g/kg
5.	Lysine	0.30	g/kg
6.	Glycine	0.20	g/kg
7.	Phenylalanine	0.20	g/kg
8.	Leucine	0.20	g/kg
9.	Isoleucine	0.20	g/kg
10.	Valine	0.20	g/kg
11.	Alanine	0.10	g/kg
12.	Histidine	0.10	g/kg
13.	Proline	0.10	g/kg
14.	Serine	0.10	g/kg
15.	Threonine	0.10	g/kg
16.	Tyrosine	0.10	g/kg
B.	**Fatty acids**		
17.	Linoleic acid	76.70	100 g D.M.
18.	Palmitic acid	39.60	100 g D.M.
19.	Linolenic acid	27.50	100 g D.M.
20.	Oleic acid	18.30	100 g D.M.
21.	Pentadecanoic acid	5.10	100 g D.M.
22.	Stearic acid	4.40	100 g D.M.
23.	Vaccenic acid	3.70	100 g D.M.
24.	Myristic acid	3.50	100 g D.M.
25.	Lauric acid	1.80	100 g D.M.
26.	Palmitoleic acid	1.40	100 g D.M.
C.	**Macro- and microelements**		
27.	Potassium	7.04–20.32	g/kg
28.	Nitrogen	3.15–6.44	g/kg
29.	Calcium	0.47–2.59	g/kg
30.	Phosphorus	0.42–1.36	g/kg
31.	Sulfur	0.39–1.64	g/kg
32.	Magnesium	0.29–1.89	g/kg
33.	Iron	6.00–171.00	mg/kg
34.	Pigments	3.43–37.25	mg/kg
35.	Zinc	3.30–41.00	mg/kg
36.	Copper	1.20–8.10	mg/kg
D.	**Dry matter**	58.50–83.00	%

2.2. Phytochemical Characterization of the Extract from Cornelian Cherry

The data obtained in this study shows that cornelian cherry contains significant levels of polyphenols (17.41 ± 0.67 mg GAE/g D.M.), flavonoids (5.64 ± 0.28 mg EC/g D.M.), and total anthocyanin content (18.27 ± 0.91 mg C3G/g D.M.), as well as a relevant antioxidant activity (185.15 ± 1.15 mMol TE/g D.M.).

The polyphenolic content was analyzed in comparison with the data available in the literature (Table 3).

Table 3. Phytochemical profiles of different cornelian cherry extracts.

Identified Compound	Extraction Method	Solvent Extraction	Results	Unit of Measurement	Reference
Total anthocyanin content	ultrasound	Ethanol 60%, ethanol 80%	189.70–261.84	mg C3G/100 g D.M	[31]
	ultrasound	Hot water	1.23 ± 0.10	mg C3G/g DW	[32]
Total polyphenolic content	ultrasound	Ethanol 60%, ethanol 80%	1700.64–4854.61	mg GAE/100 g D.M.	[31]
	ultrasound	20 mL methanol + 2% HCl	163.69 ± 0.04 (S1) to 359.28 ± 9.57 (H2)	mg GAE/100 g FW [1]	[33]
Total flavonoid content	Soxhlet method	Water Petroleum ether Methanol Acetone Ethyl actate	3.53 ± 0.39 6.91 ± 0.09 7.18 ± 0.10 8.05 ± 0.76 41.49 ± 0.57	mg Rutin/g extract	[34]
	ultrasounds	20 mL methanol + 2% HCl	12.14 ± 0.01 (S1) to 64.48 ± 0.81 (H3)	mg QE [2]/100 g FW	[33]
Total antioxidant activity	maceration/stirring	100 mL acetone	677.88 ± 19.25 (ABTS [3] assay) 628.75 ± 17.41 (FRAP [4] assay)	µmol Trolox equivalents/100 g FW	[35]
	ultrasound	20 mL methanol + 2% HCl	1.24 ± 0.01 (H1) to 2.71 ± 0.05 (H3)	mmolTrolox/100 g FW	[33]

[1] FW—fresh weight; [2] QE—Quercetin Equivalent; [3] ABTS—2,2′-azino-bis3-ethylbenzothiazoline-6-sulfonic acid; [4] FRAP—Ferric Reducing Antioxidant Power Assay.

As it can be observed, the efficiency of the extraction of the total anthocyanins, flavonoids, and polyphenols is highly dependent on the extraction method and the solvent used. The cornelian cherry extract obtained in our study showed a higher global polyphenolic content, which impacted the antioxidant activity.

2.3. Co-Microencapsulation Efficiency and Phytochemical Profile of the Powders

From Table 4, it can be observed that the variant with *L. casei* showed significantly ($p < 0.05$) higher values compared to the sample without lactic acid bacteria for encapsulation efficiency and phytochemical profile and comparable values for antioxidant activity.

The anthocyanin microencapsulation efficiency was approximatively 7% higher for the WPI-MD-LC variant, the powder showing an approximatively 16% higher content in anthocyanins and polyphenols and 9% in flavonoids. The higher content of global polyphenolic content influenced the antioxidant activity. A satisfactory co-microencapsulation of 80% was obtained for the lactic acid bacteria, which accounted for 3.80×10^9 CFU/g D.M. The higher encapsulation efficiency may be explained by the enhanced potential interactions between the whey proteins and the polyphenols due to hydrogen bonding and hydrophobic associations, which may enhance the pigment and the probiotic stability within the biopolymeric matrix.

Table 4. Microencapsulation efficiency, phytochemical content, and *L. casei* content of the powders.

Analyzed Parameter	WPI-MD	WPI-MD-LC
Anthocyanin microencapsulation efficiency (%)	82.16 ± 0.78^{b}	88.95 ± 0.42^{a}
Lacticaseibacillus casei 431® microencapsulation efficiency (%)	N.A.	80
Total anthocyanin content (mg C3G/g D.M.)	11.16 ± 0.53^{b}	12.86 ± 0.86^{a}
Total polyphenolic content (mg GAE/g D.M.)	11.17 ± 0.11^{b}	12.95 ± 0.38^{a}
Total flavonoid content (mg CE/g D.M.)	4.97 ± 0.17^{b}	5.43 ± 0.09^{a}
Total antioxidant activity (mMol TE/g D.M.)	62.53 ± 1.17^{a}	63.40 ± 0.88^{a}
Lactobacillus casei 431® (CFU/g D.M.)	N.A.	3.80×10^{9}

N.A.—not applicable; WPI-MD—microencapsulated extract from cornelian cherry fruits with whey protein isolates and maltodextrin; WPI-MD-LC—microencapsulated extract from cornelian cherry fruits with whey protein isolates, maltodextrin, and *L. casei*. For each phytochemical analysis, values for the studied powders that share different lowercase letters (^{a}, ^{b}) are statistically different at $p < 0.05$, based on the Tukey test.

A recent study used WPI, inulin, chitosan, and *Lactobacillus casei* as wall materials to protect the cornelian cherry anthocyanins. The data obtained for the phytochemical profile of the powders demonstrated that the polyphenolic content was lower than in the present study (2.66 ± 0.08 mg GAE/g D.M.). Moreover, the antioxidant activity is significantly higher in this study (15.89 ± 0.19 mM Trolox/g D.M.) compared to the reference above mentioned [36].

Vasile et al. [37] microencapsulated black bean anthocyanins with *L. casei*, chitosan, and inulin from chicory. The data obtained showed that the anthocyanins had an encapsulation effectiveness of $99.33 \pm 0.13\%$, whereas the lactic bacteria had a value of $77.42 \pm 1.34\%$. Furthermore, Colin-Cruz et al. [38] suggested that wall materials have a significant influence on the encapsulation efficiency rate. These authors used spray-drying microencapsulation of blackberry (*Rubus fruticosus*) bioactive compounds from juice in whey protein concentrate, acacia gum, and maltodextrin and reported a microencapsulation efficiency of anthocyanin ranging from 71.90 to 93.00%.

2.4. In Vitro Digestion of Anthocyanins

Due to their structural particularities and pH-dependent sensitivity, the bioavailability of anthocyanin was reported to be in the range of 0.26–1.8% [39]. In order to increase the bioaccesibility of anthocyanin, the cornelian cherry extract was microencapsulated in a biopolymeric matrix containing WPI and MD in a ratio of 1:1. Both of the freeze-dried powders exhibit a good stability of the anthocyanin content during simulated stomach digestion, proving that wall matrices can protect the anthocyanins from cornelian cherry fruits. The good stability of anthocyanin in SGJ may be explained by a glycosyl moiety bonded to anthocyanin, thus affecting the anthocyanin metabolism. Therefore, it can be assumed that the flavylium cation form of anthocyanin is the most abundant in acidic environments such as the stomach, as explained by McGhie & Walton [40].

On the other hand, during intestinal digestion, a controlled release can be observed from Table 5, with an increase of anthocyanin content of 22–25% after 120 min when compared with the beginning of digestion. This may be explained by the impact of the increased pH in the SIJ, leading to the formation of the blue quinonoidal structure generated by the loss of a proton [40]. The increase in anthocyanin content was explained by Fang [41] and is related to the higher degradation rate of anthocyanin at pH values higher than 7.0. In the neutral to alkaline range of the pH scale, the anthocyanins are metabolized to anthocyanidin by the removal of sugars in the intestine, followed by degradation to smaller

compounds such as protocatechuic acid and ferulic acid. The results obtained confirm that both of the microencapsulated powders provided a satisfactory protection of anthocyanin in the gastric environment and a slow release in the small intestine, thus improving the bioaccesibility.

Table 5. In vitro digestibility of the anthocyanins.

Time (min)	WPI-MD	WPI-MD-LC
Simulated gastric juice (SGJ)		
T0 (mg C3G/g D.M.)	$7.96 \pm 0.48^{a,B}$	$9.03 \pm 0.37^{b,A}$
T30 (mg C3G/g D.M.)	$8.25 \pm 0.74^{a,A}$	$9.14 \pm 0.89^{b,A}$
T60 (mg C3G/g D.M.)	$8.42 \pm 0.53^{a,A}$	$9.62 \pm 0.66^{ab,A}$
T90 (mg C3G/g D.M.)	$8.46 \pm 0.37^{a,B}$	$10.87 \pm 0.43^{a,A}$
T120 (mg C3G/g D.M.)	$8.74 \pm 0.47^{a,B}$	$10.90 \pm 0.21^{a,A}$
Simulated intestinal juice (SIJ)		
T0 (mg C3G/g D.M.)	$12.41 \pm 0.18^{c,A}$	$12.86 \pm 0.89^{c,A}$
T30 (mg C3G/g D.M.)	$13.22 \pm 0.56^{bc,A}$	$12.98 \pm 0.17^{c,A}$
T60 (mg C3G/g D.M.)	$13.69 \pm 0.56^{abc,A}$	$13.73 \pm 0.61^{bc,A}$
T90 (mg C3G/g D.M.)	$14.51 \pm 0.63^{ab,A}$	$15.63 \pm 0.87^{ab,A}$
T120 (mg C3G/g D.M.)	$15.09 \pm 0.96^{a,A}$	$16.15 \pm 0.78^{a,A}$

WPI-MD—microencapsulated extract from cornelian cherry fruits with whey protein isolates and maltodextrin; WPI-MD-LC—co-microencapsulated extract from cornelian cherry fruits with whey protein isolates, maltodextrin, and *L. casei*. Different lowercase letters (^{a}, ^{b}, ^{c}) indicate that the values in the same collum for SGJ and SIJ are statistically different ($p < 0.05$), while different upercase letters (^{A}, ^{B}) mean that the digestion time is the same for SGJ and SIJ but correspond to different analyzed powders, and they are statistically different at $p < 0.05$, based on the Tukey test.

The in vitro experimental results demonstrate the beneficial impact of the wall materials to preserve the bioactive compounds, particularly the anthocyanins from CM fruits. The information gathered in this study confirms that the wall materials used can successfully protect the cornelian cherry fruit's anthocyanins against internal and external factors.

2.5. Colorimetric Analysis

Color remains one of the important parameters that influences consumers' acceptance of products. The colorimetric study of the microencapsulated powders revealed a red hue because of the positive value for the a* parameter, which can be associated with the anthocyanin content. On the other hand, a significant quantity of carotenoid compounds (such as β-carotene) can be linked to a positive value for the b* parameter. The light shade of the wall materials employed—WPI, MD, and lactic acid bacteria—may all be related to the luminosity of both the samples (positive L* values) (Table 6).

Table 6. Colorimetric analysis of (co-)microencapsulated powders.

Variants	L*	a*	b*	c*	h*
WPI-MD	46.29 ± 0.15^{a}	19.20 ± 0.13^{b}	6.57 ± 0.01^{b}	20.29 ± 0.12^{b}	0.33 ± 0.00^{a}
WPI-MD-LC	44.09 ± 0.25^{b}	25.01 ± 0.65^{a}	6.89 ± 0.11^{a}	25.94 ± 0.66^{a}	0.27 ± 0.00^{b}

WPI-MD—microencapsulated extract from cornelian cherry fruits with whey protein isolates and maltodextrin; WPI-MD-LC—co-microencapsulated extract from cornelian cherry fruits with whey protein isolates, maltodextrin, and *L. casei*. For each color parameter (L*, a*, b*, c*, h*), values in the same column that do not share the same lowercase letters (^{a}, ^{b}) are statistically different at $p < 0.05$, based on the Tukey test.

The L* parameter values suggest the brightness of the powders due to the biopolymeric matrices. That may be connected to the choice of less intense wall material tones. Furthermore, the addition of lactic acid bacteria powder can be linked to the pink–red shades. Consequently, when compared, the value obtained for the a* parameter of the WPI-MD-LC powder (25.01 ± 0.65) is consistent with the results for the slightly higher content of anthocyanins (12.86 ± 0.86 mg C3G/g D.M.). According to the color wheel, which is the base of the color parameters calculation, the Hue values place both the samples into the red–yellow shades.

2.6. Viability of Lactic Acid Bacteria During Storage

During the storage period of WPI-MD-LC variant, a good stability of the viable cells was observed, with a decrease from 3.80×10^9 CFU/g to 3.26×10^9 CFU/g after 7 days, t6 2.89×10^9 CFU/g D.M. after 14 days, and to 2.53×10^9 CFU/g D.M. after 21 days. Therefore, a 66.5% viability of the probiotic was estimated during the 21 days of storage at 4 °C. The decrease in viable cells was described by a first order kinetic model, allowing us to estimate the degradation rate as 52.08 ± 4.35 days with a half time of 0.013 ± 0.0009 days. The remarkable stability of the viable cells over 21 days at 4 °C may be attributed to the prebiotic activity of both the MD and the polyphenols. For example, Oluwatosinr et al. [42] reported values for *L. plantarum* of 13% after 12 weeks of storage under refrigeration when encapsulating the probiotic with MD. Even though the prebiotic activities of polyphenols are not fully scientifically demonstrated in the literature, Medeiros Alves-Santos et al. [43] highlighted that, in animal studies, the consumption of polyphenols, especially catechins, anthocyanins, and proanthocyanidins increased the abundance of *Lactobacillus, Bifidobacterium, Akkermansia, Roseburia*, and *Faecalibacterium* spp.

A similar decreasing behavior in cell viability was reported by Milea et al. [44], who microencapsulated flavonoids from yellow onion skin with WPI, inulin, MD (2:1:1), and *L. casei*. After 21 days of storage at 4 °C in the dark, the viability of the probiotic bacteria was lower by 7.51 log CFU/g D.M. Moreover, Enache et al. [45] have demonstrated the protective effect of the wall materials (WPI, inulin, and *L. casei* or WPI, casein, and *Lactobacillus casei*) when encapsulating cornelian cherry fruit anthocyanins, even after 90 days of storage in dark conditions at 4 °C (9.58 log CFU/g D.M.). However, when considering the viable cell counts, in order to have a powder with probiotic properties, it is essential to maintain a minimum 6 log of viable cells/g by the end of the storage time.

2.7. Storage Stability of the Bioactive Compounds in the Encapsulated Powders

The microencapsulated powders were evaluated for antioxidant activity and polyphenol, anthocyanin, and flavonoid content every 7 days throughout a 28-day period of storage in dark conditions at 4 °C. The data are shown in Table 7.

According to the data shown in Table 7, a significant decrease ($p < 0.05$) was observed in the microencapsulated cornelian cherry in WPI and MD without the addition of a probiotic in total polyphenols (18%), which affected the antioxidant activity (a decrease of about 46% for the powder without the probiotic). No significant differences were observed in the flavonoid and anthocyanin content. For the co-microencapsulated powder, a good storage stability was observed in terms of polyphenols, flavonoids, and anthocyanin, whereas the antioxidant activity significantly decreased after 21 days of storage (45%). It can be suggested that in the co-microencapsulated variant, some other compounds may be responsible for the decrease in the antioxidant activity, which can be correlated with the metabolic activity of the probiotics.

Table 7. Storage stability of biologically active compounds of microencapsulated powders.

Variants	Storage Time (Days)	TAA (mMol/g D.M)	TPC (mg GAE/g D.M.)	TFC (mg CE/g D.M.)	TAC (mg C3G/g D.M.)
WPI-MD	7	$59.33 \pm 0.29^{a,A}$	$10.10 \pm 0.05^{a,B}$	$4.97 \pm 0.39^{a,A}$	$10.88 \pm 0.39^{a,A}$
	14	$55.92 \pm 0.92^{b,A}$	$9.64 \pm 0.14^{b,B}$	$4.95 \pm 0.29^{a,A}$	$10.83 \pm 0.65^{a,B}$
	21	$49.51 \pm 1.36^{c,A}$	$9.16 \pm 0.11^{c,B}$	$4.94 \pm 0.06^{a,A}$	$10.64 \pm 0.06^{a,B}$
	28	$31.81 \pm 0.41^{d,A}$	$8.28 \pm 0.04^{d,B}$	$4.86 \pm 0.25^{a,A}$	$10.15 \pm 0.12^{a,B}$
WPI-MD-LC	7	$59.49 \pm 0.25^{a,A}$	$12.70 \pm 0.08^{a,A}$	$5.08 \pm 0.13^{a,A}$	$12.23 \pm 0.80^{a,A}$
	14	$56.14 \pm 0.26^{b,A}$	$12.62 \pm 0.21^{a,A}$	$5.13 \pm 0.08^{a,A}$	$12.21 \pm 0.21^{a,A}$
	21	$49.07 \pm 0.15^{c,A}$	$12.56 \pm 0.11^{a,A}$	$5.11 \pm 0.13^{a,A}$	$12.18 \pm 0.93^{a,A}$
	28	$32.69 \pm 0.80^{d,A}$	$12.49 \pm 0.03^{a,A}$	$4.96 \pm 0.15^{a,A}$	$12.13 \pm 0.55^{a,A}$

WPI-MD—microencapsulated extract from cornelian cherry fruits with whey protein isolates and maltodextrin; WPI-MD-LC—co-microencapsulated extract from cornelian cherry fruits with whey protein isolates, maltodextrin, and *L. casei*. Different lowercase letters (^{a}, ^{b}, ^{c}, ^{d}) indicate significant differences ($p < 0.05$) for each phytochemical analysis (TAA, TPC, TFC, and TAC) of WPI-MD and WPI-MD-LC powders during storage (days), while uppercase letters (^{A}, ^{B}) indicate significant differences ($p < 0.05$) between powder variants at the same storage time (day) for each phytochemical analysis, based on ANOVA results followed by the Tukey test.

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals and Reagents

The reagents used in this study were the following: acetic acid, aluminum chloride, hydrochloric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ethanol, Folin–Ciocâlteu reagent, gallic acid, maltodextrin (dextrose equivalent (DE) of 16.5–19.5), methanol, pancreatin (Kreon), pepsin from gastric porcine, potassium acetate, and Trizma hydrochloride. The reagents were purchased from Sigma Aldrich (Taufkirchen, Germany). Sodium bicarbonate was purchased from Honeywell, Fluka (Selze, Germany), and whey protein isolate 894 (WPI) was purchased from Fonterra (Clandeboye, New Zealand). Lactic acid bacteria *Lactobacillus casei* 431® strain was purchased from Chr. Hansen (Hoersholm, Denmark). *L. casei* cell viability was performed using de Man, Rogosa, and Sharpe agar (MRS agar) purchased from Merck (Darmstadt, Germany).

3.1.2. Fresh Fruit Processing

Fresh fruits of cornelian cherry were collected from Slivna village (Galati, Romania) in September 2019 and then identified by PhD botanist biologist Țupu E. The next step was to wash with distilled water and dry with a paper towel in order to remove all impurities. Finally, the stone seeds were manually removed. The resulting samples were immediately processed by freeze-drying.

3.2. Methods

3.2.1. Phytochemical Extractions

To prevent the breakdown of bioactive components, the fruits were freeze-dried (CHRIST Alpha 1-4 LD plus, Osterode am Harz, Germany) at −42 °C under 10 Pa pressure for 48 h. The obtained material was collected and packed. After grinding, 100 g of freeze-dried fruit was mixed with 400 mL of 70% ethanol and extracted for 30 min at 40 °C, 100 W, and 40 KHz (MRC Scientific Instrument 20 L, 50 kHz, Holon, Israel). The extraction parameters (temperature, duration, ultrasonic power, solvent–solid ratio) were chosen in accordance with similar studies already published [32,44]. The hydroalcoholic extract was then centrifuged (Universal 320R, Tuttlingen, Germany) at 5000 rpm for 30 min at 4 °C. The extraction was performed twice. Concentration under vacuum at 40 °C was used to remove the solvents (AVC 2-18, Christ, UK). To prepare the microencapsulated material,

the concentrated extract was weighed, dissolved in 20 mL of ultrapure water (UW), filtered, and stored in the refrigerator at 4 °C in dark conditions.

3.2.2. Microencapsulation of the Anthocyanins

The concentrated extract of the cornelian cherry fruits was combined with the chosen wall materials to protect the anthocyanins by freeze-drying microencapsulation. For this purpose, 2 g of concentrated extract of *Cornus mas* fruit was rehydrated with 20 mL of UW for each experimental variant. One variant involved the mixture of 20 mL of extract in UW in 100 mL of MD and WPI (1:1 ratio). The same protocol was applied for co-microencapsulation, with an additional step of inoculation with 1 g of *L. casei*. The pH of both samples was settled at 4.6 to allow for good stability of probiotics and anthocyanins. The mixtures were constantly homogenized for 4 h at 650 rpm with a magnetic stirrer (IKA, Deutschland, Germany). After that, both samples were stored at −4 °C for 24 h to ensure the complete hydration of the ingredients, followed by freeze-drying (CHRIST Alpha 1-4 LD plus, Osterode am Harz, Germany) for 48 h at −42 °C and 10 Pa pressure. The resulting powders were packed in sterile bags and stored at 4 °C in a dark environment for further use.

3.2.3. Phytochemical Profile of the Extract and Freeze-Dried Powders

Total polyphenol content (TPC), total anthocyanin content (TAC), total flavonoid content (TFC), and total antioxidant activity (TAA) were quantified to determine the phytochemical profile of cornelian cherry extract and microencapsulated powders.

TPC was carried out using the Folin–Ciocalteu method according to the protocol: 15.8 mL UW, 0.2 mL of sample previously diluted 1:10, and 1 mL of the Folin–Ciocalteu reagent were thoroughly mixed and shaken. After 10 min, three milliliters of sodium carbonate (3%) were added. After 60 min in complete darkness, the samples' absorbance was measured at 765 nm using a UV-Vis Jenway spectrophotometer (OSA, UK). The results were expressed as mg gallic acid/g D.M. based on a calibration curve [45].

By mixing 0.250 mL of diluted liquid sample (1:10) with 1.25 mL UW and 0.075 microliters of sodium nitrate (5%), total flavonoid compounds were measured. After five minutes, 0.15 mL of aluminum chloride (10%) was added. After six minutes, the sample was mixed with 0.77 mL UW and 0.5 mL sodium hydroxide (1 M). The same spectrophotometer was used to instantly read the absorbance at 510 nm, and the results were expressed as mg equivalent catechins/g D.M. [45].

For total anthocyanin content (TAC), as well as for surface anthocyanin content (SAC), the same spectrophotometer was used to quickly read the absorbance at 520 nm and 700 nm at two pH values. Using two buffers (buffer 1: potassium chloride at pH 1 and buffer 2: sodium acetate at pH 4.5), TAC was calculated according to Equations (1) and (2) [46]:

$$A = (pH_1 - pH_{4.5})_{520} - (pH_1 - pH_{4.5})_{700} \quad (1)$$

$$TAC\left(Cyanidin\ 3-glucoside\ equivalents, \frac{mg}{g\ DM}\right) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l} \times \frac{V}{W} \quad (2)$$

where

A—the differences in absorbance, calculated using Equation (1);

MW—molecular weight of cyanidin-3-glucoside (449.2 g/mol);

DF—dilution factor;

ε = molar extinction coefficient for cyanidin-3-glucoside (26,900 L/(mol·cm);

l = width of the cuvette in cm;

W = sample weight (mg);

V = volume in mL;

10^3 = conversion factor from g to mg.

The total antioxidant activity of the samples had been performed using the DDPH method, earlier described by Oancea et al. [46]. Briefly, 100 microliters of sample were mixed with 3900 microliters of DPPH solution. After 90 min in dark conditions, the absorbance was measured with a spectrophotometer at 515 nm. The data were finally presented as mMol Trolox equivalent/g D.M., based on the calibration curve [46].

3.2.4. Viability of Lactic Acid Bacteria

The pour plate method, MRS-agar (De Man, Rogosa, and Sharpe medium at pH 5.7), and sterile saline (0.9 g NaCl, *w*/*v*) were used to measure the survival of lactic acid bacteria with probiotic potential. The ISO 8261 [47] method was applied to determine the quantity of live *L. casei* 431® cells. This involved counting the number of live cells, which was done on MRS-agar after 48 h at 37 °C in an aerobic environment. Colony forming units (CFU/g D.M.) were used to express the viable cell count. The initial viable cell count was 10^{11}/g D.M.

3.2.5. Microencapsulation Efficiency of the Powders (%)

The quantification of total anthocyanin content (TAC) and surface anthocyanin content (SAC) in accordance with Equation (1) was used to determine the anthocyanin encapsulation efficiency (AAE) of the two microencapsulated powders.

$$AAE(\%) = \frac{TAC - SAC}{TAC} \times 100 \quad (3)$$

For TAC protocol, methanol, acetic acid, and ultrapure water were used in a ratio of 5:0.8:4.2 (*v*/*v*/*v*), whereas ethanol and methanol were mixed in an identical ratio (*v*/*v*) to measure SAC concentration. About 200 mg of powder was mixed with 5 mL of the corresponding solvent. In order to measure the total anthocyanin content, the samples were submitted to ultrasound treatment for 15 min at 40 °C, 100 W, and 40 KHz (MRC Scientific Instrument 20 L, 50 kHz, Holon, Israel) to allow the breakup of the microparticles and the release of the anthocyanin.

Following the procedure outlined by Colin-Cruz et al. [38], the co-microencapsulation efficiency (MEE) of *L. casei* was quantified. The number of viable cells in the powder (N) and the number of viable cells in the mixture just before freeze-drying (N0), both expressed as CFU/g D.M., were used to calculate the efficiency of co-microencapsulation (%) using Equation (4) [43].

$$MEE(\%) = \frac{N}{N0} \times 100 \quad (4)$$

3.2.6. In Vitro Digestion of Anthocyanins

A static model was used to investigate the in vitro digestibility of anthocyanins using simulated gastric juice (SGJ) and simulated intestinal juice (SIJ). Briefly, in a hermetically sealed plastic flask, 500 mg of sample was mixed with 10 mL Trizma hydrochloride (0.05 M, pH = 7) and 20 mL SGJ (20 mL HCl 0.1 N and 20 g pepsin, pH = 2), followed by rapid agitation until complete homogenization. After that, each microencapsulated powder in SGJ was maintained at 37 °C in an orbital shaker for 120 min. Every 30 min, 2 mL of sample was drawn, centrifuged (at 14,000 rpm for 10 min at 4 °C), and the anthocyanin concentration was calculated in accordance with the above-mentioned methodology. After 2 h, 5 mL of SGJ was mixed with 20 mL of SIJ (20 mg pancreatin and 10 mg pepsin, pH = 7.8), and the same protocol was used to sample and measure the anthocyanin content every 30 min [46].

3.2.7. Colorimetric Analysis

A CR Konica Minolta (Japan) food colorimeter and a CIE L*a*b* system, a scientific tool, were used to analyze the color properties of the two powders. The scientific instrument's display shows three variables: L* (which represents the brightness of the examined samples) and two color characteristics, a* and b*. The parameter a* identifies shades of green (negative values) or red (positive values). The parameter b* quantifies shades of blue (negative values) or yellow (positive values), while for L*, factor values range between 0 (for black) and 100 (for white). According to the procedure outlined previously by Browning et al. [48] and Enache et al. [45], two additional coordinates (C*, h*) could be calculated.

3.2.8. Storage Stability

Using the methodologies outlined above, the following tests were carried out to quantify the storage stability over time: total anthocyanin content, total polyphenol content, total flavonoid content, and total antioxidant capacity. The two experimental variants of storage stability were examined by repeating the test procedure after 7, 14, 21, and 28 days (codified as T7, T14, T21, T28) in dark conditions at 4 °C and 50–60% relative humidity.

3.3. Statistical Analysis

Using the Microsoft Excel Package Tool, each analysis for this study was carried out in triplicate, and the data were presented as means and standard deviations. Based on the Tukey test method and the 95% confidence interval with $p < 0.05$, the statistical difference between the samples was determined.

4. Conclusions

The data presented in this study highlighted the remarkable polyphenolic content of cornelian cherry extract, obtained by ethanol–water ultrasound assisted extraction. The extract was micro- and co-microencapsulated using a combination of WPI and MD, allowing us to obtain two powders with a high content in total anthocyanin, polyphenols, and flavonoids and a strong antioxidant activity. However, when the extract was co-microencapsulated with probiotics, a higher anthocyanin encapsulation efficiency was found, together with a satisfactory level of viable cells trapped in the biopolymeric matrices. Additionally, the microbial load reached 3.80×10^9 CFU/g D.M. after freeze-drying, with a good stability during storage, suggesting a potential nutraceutical and functional effect. Additionally, the protective effect of the matrix used was demonstrated by the in vitro digestibility of the anthocyanins. Accordingly, the increased bioaccesibility of the anthocyanins in the gastrointestinal simulated media was demonstrated by the good stability in gastric conditions and a slow release in the intestinal environment. Moreover, the colorimetric analysis revealed red–yellow shades for both of the experimental variants tested due to the presence of anthocyanins (positive value for the a* parameter related to the red color) and carotenoids (positive value for the b* parameter associated with yellow shades).

Further studies will be developed in order to highlight the polyphenolic fingerprint of the extract and powders in order to have a complete picture of the potential metabolic activity of the co-microencapsulated powder on the polyphenolic profile.

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References

1. Wallace, T.C. Anthocyanins in cardiovascular disease. *Adv. Nutr.* **2011**, *2*, 1–7. [CrossRef] [PubMed]
2. Pojer, E.; Mattivi, F.; Johnson, D.; Stockley, C.S. The case for anthocyanin consumption to promote human health: A review. *Compr. Rev. Food Sci. Food Saf.* **2013**, *12*, 483–508. [CrossRef]
3. Yousuf, B.; Gul, K.; Wani, A.A.; Singh, P. Health benefits of anthocyanins and their encapsulation for potential use in food systems: A review. *Crit. Rev. Food Sci. Nutr.* **2015**, *56*, 2223–2230. [CrossRef] [PubMed]
4. Doniță, N.; Popescu, A.; Paucă-Comănescu, M.; Mihăilescu, S.; Biriș, I.A. *Habitats from Romania (ro. Habitatele din România)*; Editura Tehnicã-Silvicã: București, Romania, 2005; pp. 1–442, ISBN 973-96001-4-X.
5. Dinda, B.; Kyriakopoulos, A.M.; Dinda, S.; Zoumpourlis, V.; Thomaidis, N.S.; Velegraki, A.; Markopoulos, C.; Dinda, M. *Cornus mas* L. (cornelian cherry), an important European and Asian traditional food and medicine: Ethnomedicine, phytochemistry and pharmacology for its commercial utilization in drug industry. *J. Ethnopharmacol.* **2016**, *193*, 670–690. [CrossRef] [PubMed]
6. Ahmadipour, S.H.; Vakili, M.; Ahmadipour, S. Phytotherapy for children's nocturnal enuresis. *J. Med. Biomed. Sci.* **2018**, *6*, 23–29. [CrossRef]
7. Szczepaniak, O.M.; Kobus-Cisowska, J.; Kusek, W.; Przeor, M. Functional properties of Cornelian cherry (*Cornus mas* L.): A comprehensive review. *Eur. Food Res. Technol.* **2019**, *245*, 2071–2087. [CrossRef]
8. Enache, I.M.; Coman, G.; Rosca, S.; Vizireanu, C.; Mihalcea, L. Optimization conventional extraction of bioactive compounds from *Cornus mas* by RSM and determination of favorability factors by GIS technique. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2021**, *49*, 12307. [CrossRef]
9. Khezri, S.; Seyedsaleh, M.M.; Seyedsaleh, I.; Dastras, M.; Dehghan, P. Whey: Characteristics, Applications and Health Aspects. In Proceedings of the 3rd International Conference on Science and Engineering, Beijing, China, 8–10 July 2016; pp. 1–9.
10. Cruz-Molina, A.V.D.L.; Ayala Zavala, J.F.; Bernal Mercado, A.T.; Cruz Valenzuela, M.R.; González-Aguilar, G.A.; Lizardi-Mendoza, J.; BrownBojorquez, F.; Silva-Espinoza, B.A. Maltodextrin encapsulation improves thermal and pH stability of green tea extract catechins. *J. Food Proc. Preserv.* **2021**, *45*, e15729. [CrossRef]
11. Andersson, H.; Asp, N.G.; Bruce, Å.; Roos, S.; Wadström, T.; Wold, A.E. Health effects of probiotics and prebiotics a literature review on human studies. *Scandinav. J. Nutr./Naringsforsk.* **2001**, *45*, 58–75. [CrossRef]
12. Ciocârlan, V. *The Illustrated Flora of Romania-Pteridophyta and Spermatophyta (ro: Flora ilustrată a României-Pteridophyta et Spermatophyta)*; Editura Ceres: Bucureşti, Romania, 2009; pp. 1–1141.
13. ITIS Report. (*Cornus mas* TSN 565094)—Integrated Taxonomic Information System—Report. 2020. Available online: https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic (accessed on 5 May 2025).
14. GBIF—Global Biodiversity Information Facility. 2020. Available online: https://www.gbif.org/species/3082263 (accessed on 5 May 2025).
15. Ercisli, S.; Orhan, E.; Esitken, A.; Yildirim, N.; Agar, G. Relationships among some cornelian cherry genotypes (*Cornus mas* L.) based on RAPD analysis. *Genet. Resour. Crop Evol.* **2008**, *55*, 613–618. [CrossRef]
16. Fan, C.; Xiang, Q.Y. Phylogenetic relationships within *Cornus* (Cornaceae) based on 26S rDNA sequences. *Am. J. Bot.* **2001**, *88*, 1131–1138. [CrossRef]
17. Xiang, Q.Y.; Thomas, D.T.; Zhang, W.; Manchester, S.R.; Murrell, Z. Species level phylogeny of the genus *Cornus* (Cornaceae) based on molecular and morphological evidence. Implications for taxonomy and Tertiary intercontinental migration. *Taxon* **2006**, *55*, 9–30. [CrossRef]
18. Eyde, R.H. Comprehending Cornus: Puzzles and progress in the systematics of the dogwoods. *Bot. Rev.* **1988**, *54*, 233–351. [CrossRef]
19. Maghradze, D.; Abashidze, E.; Bobokashvili, Z.; Tchipashvili, R.; Maghlakelidze, E. Cornelian cherry in Georgia. *Acta Hortic.* **2009**, *818*, 65–72. [CrossRef]

20. Ercişli, S. Cornelian cherry germplasm resources of Turkey. *J. Fruit Ornam. Plant Res.* **2004**, *12*, 87–92.
21. Dokoupil, L.; Řeznílek, V. Production and use of the cornelian cherry—*Cornus mas* L. *Acta Univ. Agric. Silvic. Mendel. Brun.* **2012**, *60*, 49–58. [CrossRef]
22. Klimenko, S. The cornelian cherry (*Cornus mas* L.): Collection, preservation, and utilization of genetic resources. *J. Fruit Ornam. Plant Res.* **2004**, *12*, 93–98.
23. Seeram, N.P.; Schutzki, R.; Chandra, A.; Nair, M.G. Characterization, quantification, and bioactivities of anthocyanins in *Cornus* species. *J. Agric. Food Chem.* **2002**, *50*, 2519–2523. [CrossRef]
24. Yilmaz, K.U.; Ercisli, S.; Zengin, Y.; Sengul, M.; Kafkas, E.Y. Preliminary characterisation of cornelian cherry (*Cornus mas* L.) genotypes for their physico-chemical properties. *Food Chem.* **2009**, *114*, 408–412. [CrossRef]
25. Koyuncu, T.; Tosun, I.; Pinar, Y. Drying characteristics and heat energy requirement of cornelian cherry fruits (*Cornus mas* L.). *J. Food Eng.* **2007**, *78*, 735–739. [CrossRef]
26. Hassanpour, H.; Yousef, H.; Jafar, H.; Mohammad, A. Antioxidant capacity and phytochemical properties of cornelian cherry (*Cornus mas* L.) genotypes in Iran. *Sci. Hortic.* **2011**, *129*, 459–463. [CrossRef]
27. Vareed, S.K.; Reddy, M.K.; Schutzki, R.E.; Nair, M.G. Anthocyanins in *Cornus alternifolia*, *Cornus controversa*, *Cornus kousa* and *Cornus florida* fruits with health benefits. *Life Sci.* **2006**, *78*, 777–784. [CrossRef]
28. Mattera, R.; Molnar, T.; Struwe, L. *Cornus* × *elwinortonii* and *Cornus* × *rutgersensis* (cornaceae), new names for two artificially produced hybrids of big-bracted dogwoods. *PhytoKeys* **2015**, *55*, 93–111. [CrossRef]
29. Nijveldt, R.J.; van Nood, E.; van Hoorn, D.E.; Boelens, P.G.; van Norren, K.; van Leeuwen, P.A. Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425. [CrossRef]
30. Brindza, P.; Klimenko, S.V.; Grigorieva, O.; Brindza, J.; Tóth, D. Biological and commercial characteristics of cornelian cherry (*Cornus mas* L.) population in the gemer region of Slovakia. *Acta Hortic.* **2009**, *818*, 85–94. [CrossRef]
31. Dumitraşcu, L.; Aprodu, I.; Stănciuc, N. A preliminary study on improving the extraction of phenolic compounds from cornelian cherry fruits. *Ann. Univ. Dunarea De Jos Galati. Fasc. VI—Food Technol.* **2019**, *43*, 100–109. [CrossRef]
32. Enache, I.M.; Vasile, A.M.; Enachi, E.; Barbu, V.; Stănciuc, N.; Vizireanu, C. Co-microencapsulation of anthocyanins from cornelian cherry fruits and lactic acid bacteria in biopolymeric matrices by freeze-drying: Evidences on functional properties and applications in food. *Polymers* **2020**, *12*, 906. [CrossRef] [PubMed]
33. Cosmulescu, S.N.; Trandafir, I.; Cornescu, F. Antioxidant capacity, total phenols, total flavonoids and colour component of cornelian Cherry (*Cornus mas* L.) wild genotypes. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2018**, *47*, 390. [CrossRef]
34. Stankovic, M.S.; Zia-Ul-Haq, M.; Bojovic, B.M.; Topuzovic, M.D. Total phenolics, flavonoid content and antioxidant power of leaf, flower and fruits from cornelian cherry (*Cornus mas* L.). *Bulg. J. Agric. Sci.* **2014**, *20*, 358–363.
35. Moldovan, B.; Filip, A.; Clichici, S.; Suharoschi, R.; Bolfa, P.; David, L. Antioxidant activity of Cornelian cherry (*Cornus mas* L.) fruits extract and the in vivo evaluation of its anti-inflammatory effects. *J. Funct. Foods* **2016**, *26*, 77–87. [CrossRef]
36. Kalra, E.K. Nutraceutical-definition and introduction. *AAPS PharmSci* **2003**, *5*, 27–28. [CrossRef] [PubMed]
37. Vasile, M.A.; Milea, Ș.A.; Enachi, E.; Barbu, V.; Cîrciumaru, A.; Bahrim, G.E.; Râpeanu, G.; Stănciuc, N. Functional enhancement of bioactives from black beans and lactic acid bacteria into an innovative food ingredient by comicroencapsulation. *Food Bioprocess Technol.* **2020**, *13*, 978–987. [CrossRef]
38. Colín-Cruz, M.A.; Pimentel-González, D.J.; Carrillo-Navas, H.; Alvarez-Ramírez, J.; Guadarrama-Lezama, A.Y. Co-encapsulation of bioactive compounds from blackberry juice and probiotic bacteria in biopolymeric matrices. *LWT* **2019**, *110*, 94–101. [CrossRef]
39. Kim, I.; Moon, J.K.; Hur, S.J.; Lee, J. Structural changes in mulberry (*Morus Microphylla*. Buckl) and chokeberry (*Aronia melanocarpa*) anthocyanins during simulated in vitro human digestion. *Food Chem.* **2020**, *318*, 126449. [CrossRef] [PubMed]
40. McGhie, T.K.; Walton, M.C. The bioavailability and absorption of anthocyanins: Towards a better understanding. *Mol. Nutr. Food Res.* **2007**, *51*, 702–713. [CrossRef]
41. Fang, J. Bioavailability of anthocyanins. *Drug Metab. Rev.* **2014**, *46*, 508–520. [CrossRef]
42. Oluwatosin, S.O.; Tai, S.L.; Fagan-Endres, M.A. Sucrose, maltodextrin and inulin efficacy as cryoprotectant, preservative and prebiotic–towards a freeze dried *Lactobacillus plantarum* topical probiotic. *Biotechnol. Rep.* **2022**, *33*, e00696. [CrossRef]
43. Medeiros Alves-Santos, A.; Araújo Sugizaki, C.S.; Carielo Lima, C.; Veloso Naves, M.M. Prebiotic effect of dietary polyphenols: A systematic review. *J. Funct. Foods* **2020**, *74*, 104169. [CrossRef]
44. Milea, Ș.A.; Vasile, M.A.; Crăciunescu, O.; Prelipcean, A.-M.; Bahrim, G.E.; Râpeanu, G.; Oancea, A.; Stănciuc, N. Co-microencapsulation of flavonoids from yellow onion skins and lactic acid bacteria lead to multifunctional ingredient for nutraceutical and pharmaceutics applications. *Pharmaceutics* **2020**, *12*, 1053. [CrossRef]
45. Enache, I.M.; Vasile, M.A.; Crăciunescu, O.; Prelipcean, A.M.; Oancea, A.; Enachi, E.; Barbu, V.V.; Stănciuc, N.; Vizireanu, C. Co-microencapsulation of anthocyanins from cornelian cherry (*Cornus mas* L.) fruits and lactic acid bacteria into antioxidant and anti-proliferative derivative powders. *Nutrients* **2022**, *14*, 3458. [CrossRef] [PubMed]
46. Oancea, A.-M.; Hasan, M.; Vasile, A.M.; Barbu, V.; Enachi, E.; Bahrim, G.; Râpeanu, G.; Silvi, S.; Stănciuc, N. Functional evaluation of microencapsulated anthocyanins from sour cherries skins extract in whey proteins isolate. *LWT* **2018**, *95*, 129–134. [CrossRef]

47. *ISO 8261 IDF122:2001*; Milk and Milk Products—General Guidance for the Preparation of Test Samples, Initial Suspensions and Decimal Dilutions for Microbiological Examination. ISO: Geneva, Switzerland, 2001.
48. Browning, W.D.; Contreras-Bulnes, R.; Brackett, M.G.; Brackett, W.W. Color differences: Polymerized composite and corresponding Vitapan Classical shade tab. *J. Dent.* **2009**, *37*, 34–39. [CrossRef] [PubMed]

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Influence of β-Cyclodextrin on the Overall Antioxidant Activity and DPPH· Reaction Kinetics of Fresh Raspberry (*Rubus idaeus* L.) and Dehydrated Strawberry (*Fragaria* × *ananassa* Duch.) Extracts

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Abstract

The influence of natural β-cyclodextrin (β-CD) on the overall antioxidant activity of berry extracts is presented in this study. Raw raspberry (*Rubus idaeus* L.) and β-CD-assisted dehydrated strawberry (*Fragaria* × *ananassa* Duch.) ethanolic extracts (RB and SB, respectively) were spectrophotometrically monitored in the presence of 1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH·) solution in the absence or presence of β-CD. Cyanidin 3-*O*-glucoside (Cy3G) was used as standard compound, being identified by RP-HPLC in both RB and SB at 14.62 and only 0.15 mg/100 g fresh weight (fw). Pelargonidin 3-*O*-glucoside (Plg3G) was the most concentrated anthocyanin in SB (estimated at 2.46 mg/100 g fw). Higher antioxidant activities (expressed as the radical scavenging activity, RSA, %) were obtained for SB dehydrated in the presence of β-CD. The RSA values increased by 35% in comparison with the SB dehydrated by the classical method. On the other hand, the DPPH· reaction kinetic parameters significantly differed for RB extracts evaluated in the presence of 1 mM β-CD (in water). The DPPH· reaction rate in the 3–15 min time range was 25% higher for the RB extracts obtained from the β-CD-assisted dehydrated samples. This study demonstrates for the first time the protection capacity of β-CD against the degradation of antioxidants during the classical dehydration process of berries. This technology can be extended to other fruits and scaled up for obtaining high-quality fruit-based products.

Keywords: antioxidants; anthocyanin; cyanidin 3-*O*-glucoside; fruit extracts; cyclodextrin assisted dehydration; cyclodextrin assisted DPPH· method; reaction kinetics

1. Introduction

Natural antioxidants are some of the most important biologically active compounds in plants. They perform various biological activities, such as anti-aging, anti-cancer, antidiabetic, anti-inflammatory, anti-microbial, hepatoprotective, nephroprotective, neuroprotective, or anti-cardiovascular disease activities [1–3]. However, specific natural antioxidants have specific action mechanisms that are involved in these biological activities. They are often related to the radical scavenging activity (RSA). This is the case of the anti-aging effects of curcuminoids, anti-cancer properties of catechins, stilbenoids, and anthocyanins [1,4,5]. Among the mechanisms related to radical scavenging or inducing antioxidant enzymes, other molecular mechanisms that imply antioxidants have been demonstrated [4,6–17].

Raspberry (RB) and strawberry (SB) are widely consumed fruits, either fresh or in processed food products. They are especially reach in anthocyanin antioxidants such as cyanidin 3-*O*-glucoside (Cy3G) in fresh SB (up to 280 μg/g in *Fragaria vesca* L., wild SB, and up to 180 μg/g for *Fragaria* × *ananassa* Duch. cultivars) [18–21]. Cy3G has the highest antioxidant power (AP) among anthocyanins and anthocyanidins in SB [18]. Polyphenolic compounds in RBs belong to anthocyanins, flavonols, flavones, flavanols, flavanones, catechins, isoflavonoids, hydroxycinnamic acids, as well as ellagitannins and ellagic acid [22–24]. The total phenolic content (TPC) depends on various factors including the cultivar, ripening, growing parameters, or storage conditions. TPC values reach 6.45 mg/g fw, depending on the cultivar. However, the mean TPC values are generally four to five times lower [24]. Among anthocyanins, cyanidin glycosides are the most important. Total anthocyanin content (TAC) can reach 1.16 mg Cy3G/g fw [25–28].

The anthocyanin stability in processed or stored berry fruits is consistently low, especially at higher temperatures, oxidative conditions and the presence of light. The degradation of pelargonidin 3-*O*-glucoside (Plg3G) at high and low temperatures revealed half-time values up to 137 min at 100 °C and 80 days at 25 °C [18]. The total loss of anthocyanins from Bordo grape extracts is almost 89% in the liquid extract and 48% in the freeze-dried extract. The degradation of anthocyanins is significantly reduced (16.5–30%) in maltodextrin-based spray-dried samples [29]. The stability of cyanidin 3-*O*-rutinoside in black RB pomace extracts is very low, especially during the storage at 25 and 40 °C in water (residual content less than 5%). However, the stability is significantly increased for extracts obtained with natural deep eutectic solvents (NaDESs) of about 90 and 50% at 25 °C and 40 °C, respectively [30]. The stability of anthocyanins and the other antioxidants strongly depends on the processing parameters of berry fruits, including extraction, advanced non-thermal (chilled or frozen berries, preservation by pulsed light treatments, high-pressure processing, etc.), and especially thermal processing of berry fruits such as drying and dehydration, pasteurization, cooking, canning, fermentation for obtaining purée, juice, concentrate and syrup, jelly, jam, or dried berries [27,31].

Drying and dehydration are widely used for fruit preservation. Both processes remove moisture/water from fruits. Dehydration generally removes up to 70–90% moisture from the fresh fruits and, consequently, the shelf life is only few months. On the other hand, drying removes moisture down to a content inhibiting bacteria and mold development. The shelf life of the dried products is many years, such as in the case of spray-drying or freeze-drying [32–35]. Various dehydration and drying methods were used for extending the shelf life of fruits. The classical ones are air-drying, sun and solar-tunnel drying, microwave-drying, and freeze- and spray-drying. For enhancing performance and reducing degradation, combined dehydration and drying techniques, as well as pretreatments, were used (microwave vacuum-drying/microwave multi-flash drying, microwave-vacuum drying, freeze-drying combined with the far-infrared drying, blanching, osmotic pretreatment, immersing in various solutions) [32]. Convective or osmotic dehydration (ultrasound-

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assisted in some cases) under high hydrostatic pressures were used for the dehydration of SB [34,36,37]. Drying SB and RB involved classical convective, infrared, solar, freeze- and spray-drying, or combined techniques [38–44].

Stabilization and extending of the SB and RB shelf life can be performed using additives obtained by micro- and nanoencapsulation or biofilm coatings before or after processing [45–48]. Natural or modified cyclodextrins (CDs) were widely used in this regard. They are cyclic oligosaccharides containing six to eight α-D-glucopyranose units, having molecular encapsulation, controlled release, enhanced stability, and apparent water solubility properties of hydrophobic biologically active compounds. Thus, edible biofilms based on *Cryptococcus laurentii* (Kuff) Lodder for the inhibition of the mold growth, in combination with alginate, fatty acids, fatty acid monoglycerides, and β-cyclodextrin (β-CD), were used for coating the SBs, with improved quality and storage properties [49]. Chitosan/polyvinyl alcohol film loading hop β-acids/β-CD inclusion complex was used for extending the shelf life of the SBs [45]. Nerolidol/hydroxypropyl-β-CD inclusion complex nanofibers were used as ingredient for obtaining active SB packaging [48]. An antibacterial hydrogel based on κ-carrageenan, and carvacrol/hydroxypropyl-β-CD composite for SB preservation, was successfully applied [46]. A coating formulation based on whey protein, glycerol, lactose, and β-CD in post-drying and freeze-drying of SB pieces was also used [50]. RB preservation was performed by improving the performance of the konjac glucomannan film with β-CD/eugenol-based nanoemulsion [51].

The use of CDs during the processing of SB or RB such as dehydration or radical scavenging extracts have not yet been studied. This study aimed to evaluate for the first time the influence of β-CD on the convective air-dehydration of the β-CD pretreated SB (*Fragaria* × *ananassa* Duch.) slices and on the RSA of antioxidants/anthocyanins from RB (*Rubus idaeus* L.) extracts through the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) kinetic parameters values. The mentioned substance was expected to modulate the overall antioxidant activity, rate constant, and half-time values during the reaction of SB and RB antioxidants with DPPH· and reveal the importance of using this natural and GRAS-approved additive for enhancing the quality of the processed berry fruits.

2. Results

2.1. Obtaining the Berry Extracts and Quantification of Anthocyanins

SB and RB extracts were obtained by semi-continuous Soxhlet extraction using well-grounded fresh fruits and ethanol as a solvent at a mass–volume ratio of 1:12. The extraction cycles were at least five. However, the multiple extraction continued until no color was observed for the last cycle. Thus, nine and five cycles were performed for obtaining the SB and RB extracts, respectively. The raw extract volumes were 297.1 ± 0.2 mL. For a proper comparison, every extract was completed with ethanol to the final volume of 300 mL.

Quantification of the main anthocyanins in SB and RB extracts was performed using the calibration curves for Cy3G and cyanidin, Cy (Equations (1) and (2) and Figures S1 and S2 in the Supplementary Materials file), which had limit of detection (LOD) and limit of quantitation (LOQ) values of 2.54 and 4.23 μg/mL for Cy3G, respectively, 1.31 and 2.18 μg/mL for Cy (see Section 4.4). The calibration curve for Cy3G was also used for the quantification of the total anthocyanin and anthocyanidin content (as mg Cy3G/100 g), considering all HPLC peaks identified at 525 nm.

$$\begin{gathered}\text{Conc.}_{\text{Cy3G}}\ (\text{mg/mL}) = 0.001692(\pm 0.000033)\cdot \text{Area}_{\text{HPLC(Cy3G)}} \\ n = 6,\ r = 0.9990,\ r^2 = 0.9981,\ F = 2589,\ p < 10^{-5},\ s = 0.015\end{gathered} \quad (1)$$

$$\begin{gathered}\text{Conc.}_{Cy}\ (\text{mg/mL}) = 0.000651(\pm 0.000007)\cdot \text{Area}_{HPLC(Cy)} \\ n = 7,\ r = 0.9997,\ r^2 = 0.9994,\ F = 9246,\ p < 10^{-5},\ s = 0.008\end{gathered} \quad (2)$$

Representative HPLC chromatograms for the fresh SB and RB extracts are presented in Figures 1 and 2 (multiplicate samples). In the upper right corner of these figures are presented the HPLC chromatograms for the standard anthocyanins and anthocyanidins (Cy3G and Cy). However, the most concentrated anthocyanin in SB extracts was Plg3G, which was quantified using the same Cy3G standard curve [18,20,52]. Thus, the Cy3G content in fresh SB and RB was 14.62 ± 2.64 and only 0.32 ± 0.08 mg/100 g fw, while the total anthocyanin and anthocyanidin contents were 73.18 ± 12.39 and 4.28 ± 0.56 mg Cy3G/100 g (Tables 1 and 2). In both extracts, the Cy content was significantly lower (0.64 ± 0.07 and 0.017 ± 0.003 mg/100 g fw, respectively).

Table 1. Cyanidin 3-*O*-glucoside (Cy3G), cyanidin (Cy), and total anthocyanin and anthocyanidin content (as Cy3G) in fresh raspberries (values are expressed as mean ± SD, n = 5).

Anthocyanins and Anthocyanidins	Content (mg/100 g fw)
Cyanidin 3-*O*-glucoside	14.62 ± 2.64
Cyanidin	0.64 ± 0.07
Total anthocyanins and anthocyanidins [1]	73.18 ± 12.39

[1] Total anthocyanin and anthocyanidin content was determined by HPLC (all antioxidant compounds separated and identified at 525 nm were counted and the content was expressed as mg cyanidin 3-*O*-glucoside/100 g fw).

Table 2. Cyanidin 3-*O*-glucoside (Cy3G), pelargonidin 3-*O*-glucoside (Plg3G), cyanidin (Cy), and total anthocyanin and anthocyanidin content (as mg Cy3G/100 g fw) in fresh strawberries (values are expressed as mean ± SD, n = 2).

Anthocyanins and Anthocyanidins	Content in Fresh Fruits (mg/100 g fw)	Content in β-CD-Assisted Dehydrated Fruits (mg/100 g) [1]
Cyanidin 3-*O*-glucoside	0.32 ± 0.08	0.15
Pelargonidin 3-*O*-glucoside [2]	2.46 ± 0.38	1.64
Cyanidin	0.017 ± 0.003	0.033
Total anthocyanins and anthocyanidins [3]	4.28 ± 0.56	4.30

[1] Single determination. [2] Pelargonidin 3-*O*-glucoside was identified according to [18–20] and was quantified as mg Cy3G/100 g. [3] Total anthocyanin and anthocyanidin content was determined by HPLC (all antioxidant compounds separated and identified at 525 nm were counted, and the content is expressed as mg Cy3G/100 g).

2.2. Dehydration of Strawberry Slices with or Without β-Cyclodextrin as an Additive

Dehydration was only applied for SB slices. SBs are most often subjected to dehydration in comparison with other berries such as RB [42,53]. Generally, RBs are subjected to drying at significantly lower moisture content and do not fit the scope of the present study [44,54].

β-CD-assisted dehydration was applied for the first time and consisted of uniformly distributing the β-CD powder on the surface of SB slices. The retained amount of β-CD was determined as the difference between the starting amount of β-CD (excess) and the remaining amount that was not retained by the fruit slices. Approximately 1.4–1.5% of β-CD powder (related to the fresh fruit slices) was used for the dehydration process. Classical convective air-dehydration was applied.

SB dehydration reduced the mass of the fruit slices by approximately ten times. The total moisture removed from the SB slices (without β-CD) was 90.56%, while the β-CD-assisted dehydration removed 90.31% of the moisture from the fresh SB slices (see Section 4.2 for further details).

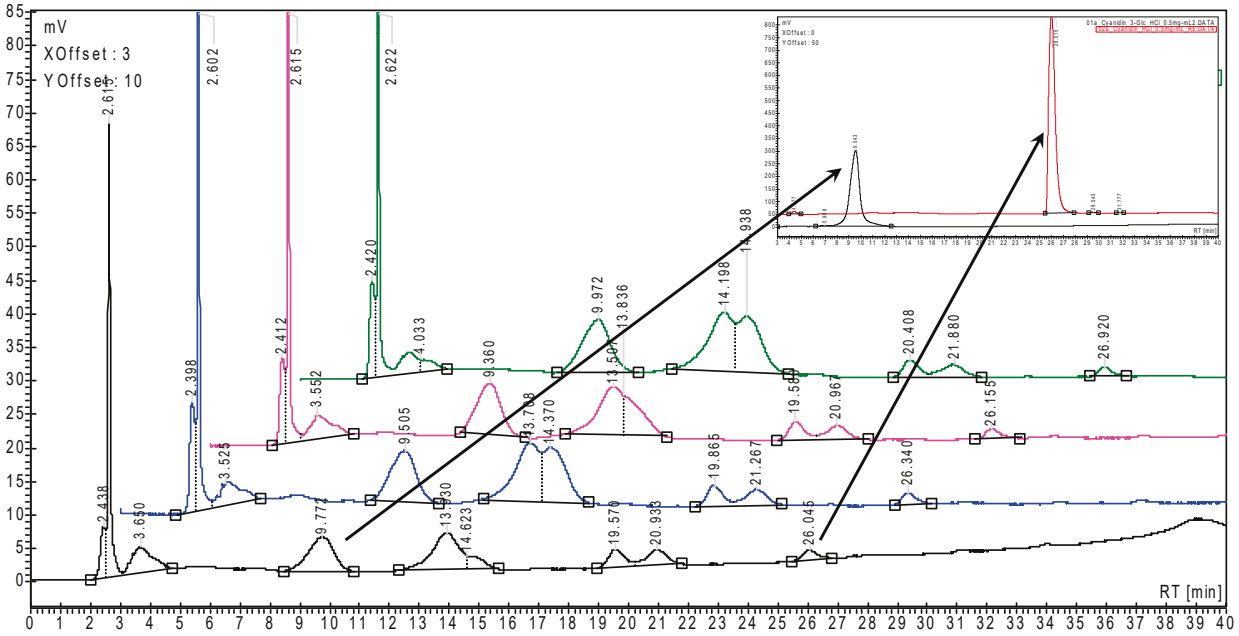


Figure 1. Superimposed of the representative HPLC chromatograms for the raspberry extracts (multiplicate samples). Cyanidin 3-*O*-glucoside (left arrow) and cyanidin (right arrow) were identified and quantified using the corresponding calibration curves obtained for the standard compounds by HPLC—upper right corner.

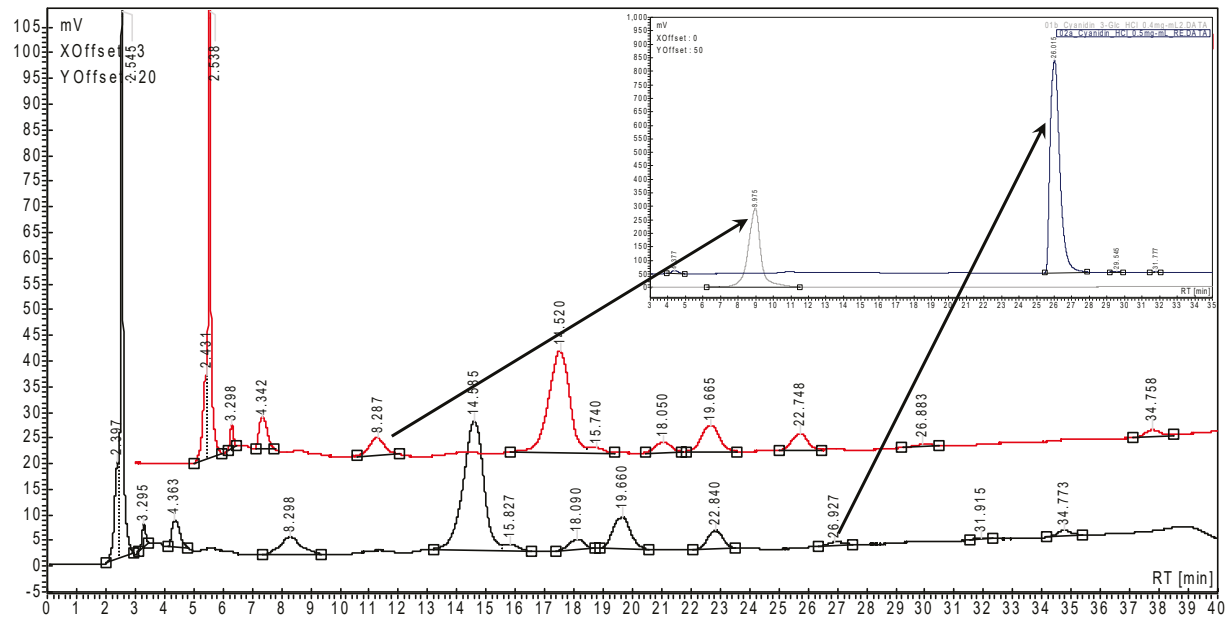


Figure 2. Superimposed of the representative HPLC chromatograms for the strawberry extracts (duplicate samples). Cyanidin 3-*O*-glucoside (left arrow) and cyanidin (right arrow) were identified and quantified using the corresponding calibration curves obtained for the standard compounds by HPLC—upper right corner. Pelargonidin 3-*O*-glucoside was identified (tentatively) at RT ~14.5 min according to [18–20].

Interestingly, only the β-CD-assisted dehydrated SBs reveal significant anthocyanin content. For β-CD-assisted dehydrated SB slices, the Cy3G content was 0.15 mg/100 g and the Plg3G content was 1.64 mg (as Cy3G)/100 g. On the contrary, the cyanidin aglycone content significantly increased at 0.033 mg/100 g after dehydration at 70 °C (Table 2).

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2.3. Antioxidant Activity of the Berry Extracts from the Raw and Dehydrated Fruits

The overall antioxidant activity of the berry extracts was evaluated using the actual RSA values during the reaction of the complex mixture of antioxidant compounds in extracts with the DPPH· radical. This free radical mimics the free radicals in the human body. These complex reactions were spectrophotometrically monitored at the maximum absorption of the DPPH· radical, i.e., 517 nm. However, some anthocyanins also absorb at this wavelength, but their absorption values at the concentration used are significantly lower than for the DPPH· radical solution (with molar absorption of $\varepsilon_{DPPH\cdot} = 1.25 \cdot 10^4$ L·mol^{-1}·cm^{-1} in ethanol [55,56]; see Figures S3–S8 in the Supplementary Material file). Consequently, this absorbance was subtracted from the DPPH· absorbance values during monitoring.

The variation in the RSA values in time has a logarithmic behavior, especially at high dilutions (Figures 3 and 4, Figures S9–S13 in the Supplementary Materials file). For comparison, similar studies were performed for the standard 0.4 mM Cy3G solutions, not assisted or β-CD assisted during spectrophotometric monitoring (Figure 5 and Figure S14 in the Supplementary Materials file). In all cases, the DPPH· reaction is very rapid in the first time range of approximately 30 s, followed by a slower reaction for the next time range (up to approximately 3 min). Then, the DPPH· reaction becomes much slower, even after the monitoring period of 15 min. Consequently, three time values were selected (i.e., ½, 3 and 15 min) for comparing the RSA values. The mean RSA values for the extracts obtained from the fresh berries were 44.84 and only 18.22% for the SB and RB extracts at ½ min, while these values significantly increased to 67.80 and 46.96, respectively, 81.17 and 72.93% for 3 and 15 min (Tables 3 and 4). In the case of RB extracts, these values are comparable with the RSA values for the standard 0.4 mM Cy3G solution (20.92, 37.76, and 66.37% at ½, 3 and 15 min, respectively, Table 5).

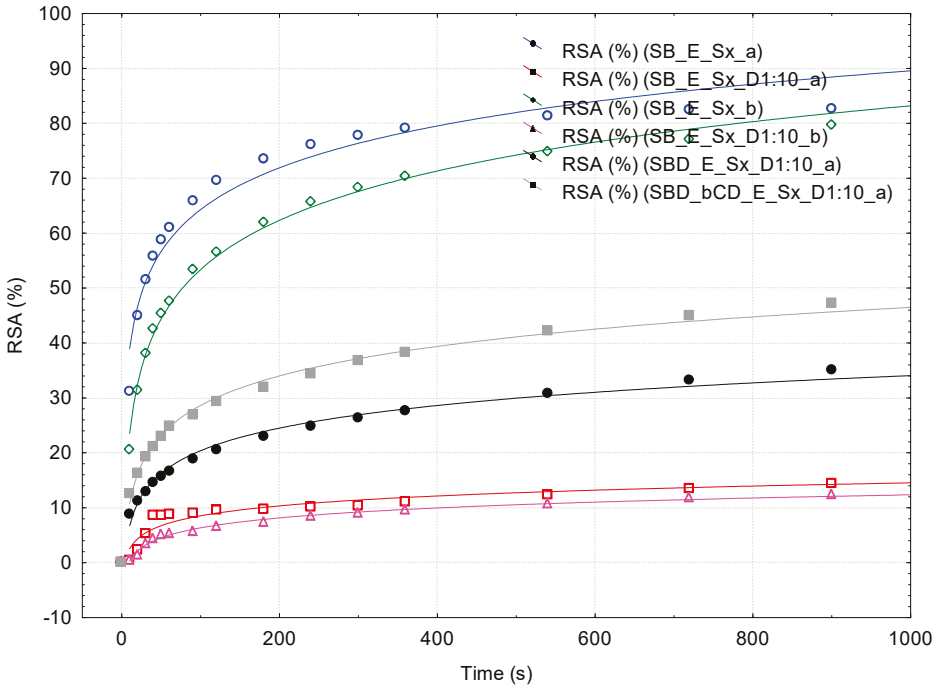


Figure 3. Antioxidant activity of the fresh strawberry extracts (duplicates; blue circle and green diamond), 1:10 diluted fresh strawberry extracts (duplicates; red square and pink triangle), and 1:10 diluted extracts obtained from the dehydrated strawberries, without (black circle) and with β-cyclodextrin (gray square) as an additive. Antioxidant activity is expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min. Logarithmic correlations are also provided.

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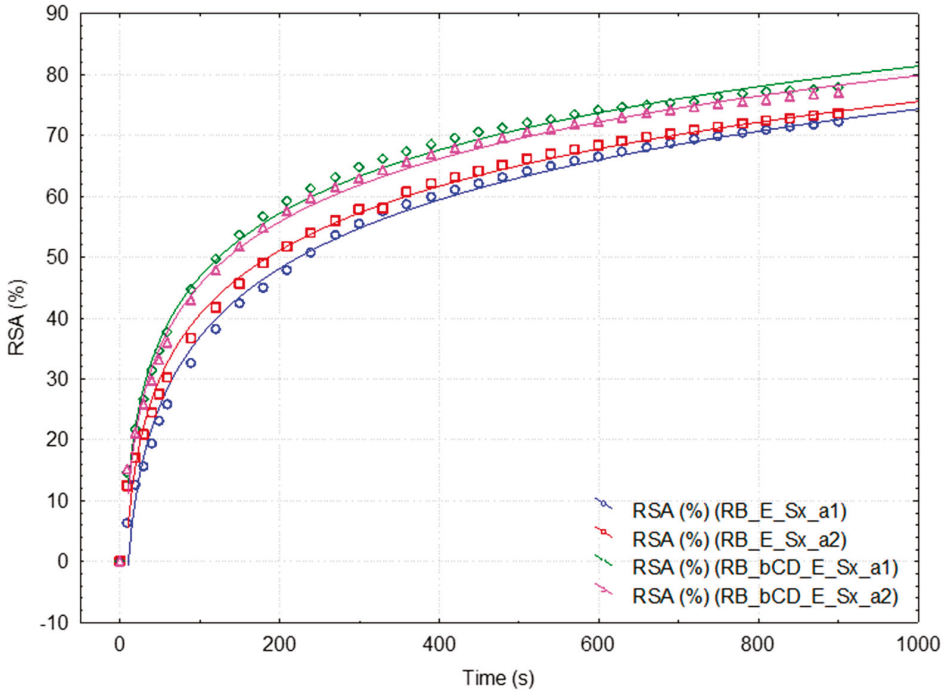


Figure 4. Antioxidant activity of the fresh raspberry extracts, β-cyclodextrin assisted (duplicates; green diamond and pink triangle) or not assisted (duplicates; blue circle and red square), during spectrophotometric monitoring. Antioxidant activity is expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min. Logarithmic correlations are also provided.

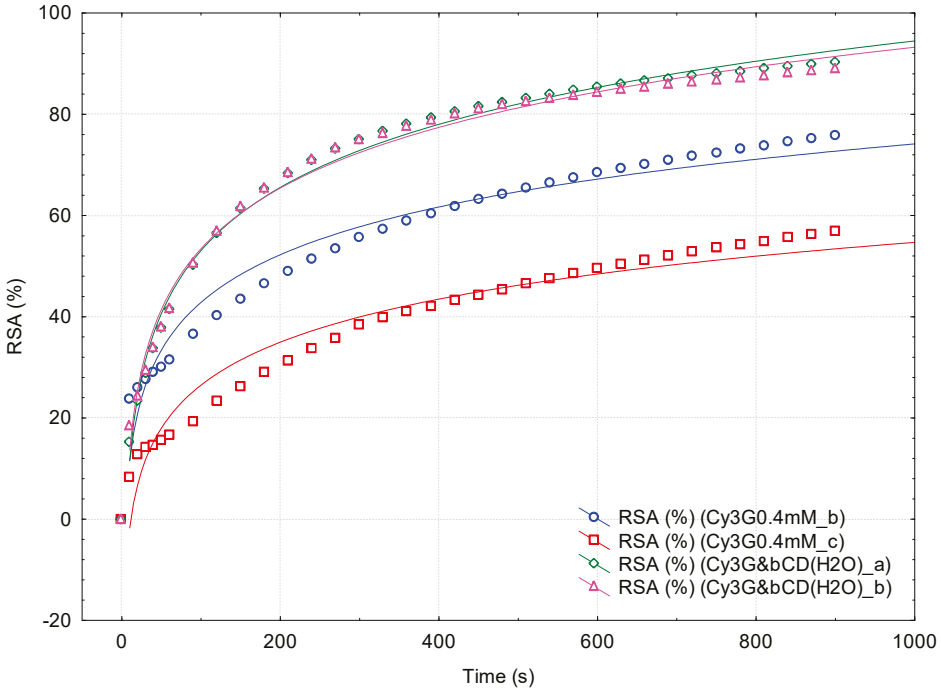


Figure 5. Antioxidant activity of the standard 0.4 mM cyanidin 3-*O*-glucoside, β-cyclodextrin assisted (green diamond and pink triangle) or not assisted (blue circle and red square), during spectrophotometric monitoring (duplicate samples). Antioxidant activity is expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min. Logarithmic correlations are also provided.

The extracts obtained from the dehydrated berries have significantly higher RSA values, especially for the β-CD-assisted samples at ½ and 3 min (between 81.47 and 84.32%, Table 3). The antioxidant activity of the raw extracts of the dehydrated SBs was significant

even at the beginning of the spectrophotometric monitoring. Consequently, the 1:10 dilution of the extracts were analyzed in the same way. The RSA values decreased at 4.37% at ½ min for the SB diluted extract, while the corresponding values for extracts obtained from the β-CD-assisted or not-assisted dehydrated samples were significantly higher (19.19 and 13.04%, $p < 0.01$). At the end of the spectrophotometric monitoring, the differences were also significant (47.32% for the case of β-CD-assisted dehydrated SB, 35.14% for the not-assisted dehydrated SB and only 13.40% for the diluted extract obtained from the fresh SB, $p < 0.01$, Table 3).

The influence of β-CD on the overall antioxidant activity was observed during spectrophotometric monitoring. The RSA values are 43.5, 18.5, and 6.1% higher for fresh RB extracts with β-CD-assisted spectrophotometric monitoring at ½, 3 and 15 min, respectively (Table 4). This behavior was also observed for the standard 0.4 mM Cy3G solution. The RSA values were 35% higher for the RB extract analyzed in the presence of β-CD in comparison with the not-assisted samples (Table 5).

Table 3. Representative Radical Scavenging Activity (RSA) values for the fresh strawberry extracts, 1:10 diluted fresh strawberry extracts (SB and SB_d1:10), and undiluted and 1:10 diluted extracts obtained from the dehydrated strawberries without (SBD and SBD_d1:10) and with β-cyclodextrin (SBD_bCD and SBD_bCD_d1:10) as an additive. For undiluted samples, RSA values with different superscript capital letters are significantly different according to Tukey's HSD (honestly significant difference) test ($p < 0.01$; on the same column). For 1:10 diluted samples, RSA values with different superscript lowercase letters are significantly different according to Tukey's HSD test ($p < 0.01$; on the same column).

Sample Code	RSA (%) (@ ½ min)	RSA (%) (@ 3 min)	RSA (%) (@ 15 min)
SB	44.84 ± 9.59^{A}	67.80 ± 8.10^{A}	81.17 ± 2.20^{A}
SB_d1:10	4.37 ± 1.31^{a}	8.60 ± 1.68^{a}	13.40 ± 1.54^{a}
SBD *	81.47^{A}	82.46^{A}	82.53^{A}
SBD_d1:10 *	13.04^{b}	22.96^{b}	35.14^{b}
SBD_bCD *	83.98^{A}	84.32^{A}	84.45^{A}
SBD_bCD_d1:10 *	19.19^{c}	31.97^{c}	47.32^{c}

* Single determination.

Table 4. Representative Radical Scavenging Activity (RSA) values for the fresh raspberry extracts not assisted (RB) or β-cyclodextrin assisted (RB_bCD) during the spectrophotometric monitoring (values are presented as mean ± standard deviation, SD, for duplicate samples). In the same column, RSA values with different superscript lowercase letters are significantly different according to Tukey's HSD test ($p < 0.1$ for the RSA values at ½ and 3 min, respectively, $p < 0.05$ for the RSA values at 15 min).

Sample Code	RSA (%) (@ ½ min)	RSA (%) (@ 3 min)	RSA (%) (@ 15 min)
RB	18.22 ± 3.67^{a}	46.96 ± 2.83^{a}	72.93 ± 0.99^{c}
RB_bCD	26.15 ± 0.52^{b}	55.67 ± 1.31^{b}	77.41 ± 0.58^{d}

Table 5. Representative Radical Scavenging Activity (RSA) values for the standard 0.4 mM cyanidin 3-*O*-glucoside not assisted (Cy3G0.4mM) or β-cyclodextrin assisted (Cy3G&bCD) during the spectrophotometric monitoring (values are presented as mean ± standard deviation, SD, for duplicate samples). In the same column, RSA values with different superscript lowercase letters are significantly different according to Tukey's HSD test ($p < 0.1$ for the RSA values at 3 min, respectively, $p < 0.15$ for the RSA values at 15 min).

Sample Code	RSA (%) (@ ½ min)	RSA (%) (@ 3 min)	RSA (%) (@ 15 min)
Cy3G0.4mM	20.92 ± 9.42^{a}	37.76 ± 12.41^{a}	66.37 ± 13.37^{c}
Cy3G&bCD	29.13 ± 0.49^{a}	65.35 ± 0.21^{b}	89.63 ± 0.90^{d}

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2.4. Kinetics of the DPPH· Reaction with Antioxidant Compounds in Berry Extracts with or Without β-Cyclodextrin as an Additive

DPPH· reaction kinetics can provide information on the overall differences between β-CD-assisted or not-assisted dehydration, respectively, and spectrophotometric monitoring (β-CD can modulates the DPPH· reaction with various antioxidant compounds). Both DPPH· reaction constant, k_n, and half-time, $t_{1/2}$, are important in this regard. Due to the very complex reactions of DPPH· radical with antioxidant compounds in extracts (anthocyanins, anthocyanidins, flavonoids, hydroxycinnamic acids, or ellagitannins), with higher concentrations compared to DPPH·, the kinetic models were only related to DPPH· [57,58]. The DPPH· also reacts with various species in the case of Cy3G (more reaction sites on the Cy3G, as well as further reaction with the products having antioxidant activity) [59,60].

All kinetic results are presented in the Supplementary Materials file. The linear correlation of the integrated reaction rate law on the selected time ranges of 0–30 s and 30–180 s ("fast" and "slow" reactions [61]; Figures S15–S20 in the Supplementary Materials file) allows determining the reaction rate constants and half-times for the zeroth- to fourth-(pseudo-)order kinetic models (Tables S1–S6 in the Supplementary Materials file; tables also contain the determination coefficients, r^2, for the integrated reaction rate law correlations). Generally, the best results for the berry extracts were obtained for the higher-(pseudo-)order kinetic models (third- and fourth-order kinetic models). On the contrary, better statistical parameters for the standard antioxidant compound Cy3G were obtained for the first- and second-(pseudo-)order kinetic models.

Representative third-(pseudo-)order kinetic models for the "fast" and "slow" DPPH· reaction with antioxidant compounds in extracts obtained from the fresh and dehydrated SB (without or with β-CD as additive) are presented in Figure 6 and Equations (3)–(5). All kinetic data for these samples are presented in the Figures S15 and S16, respectively; Tables S1 and S2 from the Supplementary Materials file. For the third-(pseudo-)order kinetic model, both dehydrated samples reveal higher rate constant of 0.794 and 0.485 $mM^{-2}{\cdot}s^{-1}$, three to five times higher than the corresponding rate constant for the fresh SB extract in the case of "fast" reaction. On the other hand, the half-time values are in the range of 1.4–7.6 min, significantly lower for the β-CD-assisted dehydrated SB samples ($p < 0.05$). These aspects can also be observed for the "slow" reaction. However, better statistical parameters were obtained for the third-(pseudo-)order kinetic models (r^2 in the range of 0.96–0.97 for the dehydrated SB samples, Equation (5)). The rate constant and half-time values for the dehydrated samples are in the range of 0.108–0.188 $mM^{-2}{\cdot}s^{-1}$ and 6.1–10.6 min, significantly different from the fresh SB samples case (k_3 = 0.024 $mM^{-2}{\cdot}s^{-1}$ and $t_{1/2(3)}$ = 47.6 min, $p < 0.001$ and $p < 0.01$, respectively).

Kinetic parameters were significantly enhanced for the RB samples, especially for the β-CD-assisted samples during spectrophotometric monitoring (Figures S17 and S18, respectively, Tables S3 and S4 in the Supplementary Materials file). For the "fast" reaction, the rate constant and half-time values of the β-CD-assisted and not-assisted RB samples were 0.590 and 0.358 $mM^{-2}{\cdot}s^{-1}$, respectively, 54.5 and 89.8 s. For the "slow" reaction, the corresponding kinetic parameters were different, but not such as in the case of SB samples. On the other hand, the statistical parameters for the kinetic models are significantly enhanced (r^2 = 0.99994 for the third-(pseudo-)order "slow" reaction model in the case of the β-CD-assisted RB sample; Figure 7 and Equations (6) and (7)). The statistical parameters for the case of the "slow" reaction of DPPH· with the standard 0.4 mM Cy3G solution in the presence of β-CD are similar, but for a lower reaction order (r^2 = 0.99991 for the second-(pseudo-)order reaction kinetics; Figure 8 and Equations (8)–(10)). The rate constant is two times higher for the β-CD-assisted RB sample, while the half-time value is 50% from the value corresponding to the not-assisted RB case (1.7 and 3.4 min, respectively). All

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results for the kinetic studies related to the reaction of DPPH· with the standard Cy3G solutions, β-CD-assisted or not-assisted during spectrophotometric monitoring, are presented in Figures S19 and S20, respectively; Tables S5 and S6 from the Supplementary Materials file.

$$\frac{1}{[C]^2} - \frac{1}{[C]_0^2} = 2 \cdot k_3 \cdot t;\ t_{1/2(3)} = \frac{3}{2} \cdot \frac{1}{k_3 \cdot [C]_0^2} \quad (3)$$

$$y = 2.86 + 0.794(\pm 0.165) \cdot x(\text{“fast” reaction for the β-CD dehydrated SB extract}) \quad r = 0.959, r^2 = 0.920, F = 23.1, p < 0.05, s = 3.69 \quad (4)$$

$$y = 22.00 + 0.188(\pm 0.018) \cdot x(\text{“slow” reaction for the β-CD dehydrated SB extract}) \quad r = 0.978, r^2 = 0.957, F = 111.0, p < 0.0002, s = 2.34 \quad (5)$$

$$y = 1.019 + 0.590(\pm 0.059) \cdot x(\text{“fast” reaction for the β-CD-assisted RB extract case}) \quad r = 0.990, r^2 = 0.980, F = 99.8, p < 0.01, s = 1.32 \quad (6)$$

$$y = 4.314 + 0.467(\pm 0.001) \cdot x(\text{“slow” reaction for the β-CD-assisted RB extract case}) \quad r = 0.99997, r^2 = 0.99994, F = 1019, p < 0.00001, s = 0.21 \quad (7)$$

$$\frac{1}{[C]} - \frac{1}{[C]_0} = k_2 \cdot t;\ t_{1/2(3)} = \frac{1}{k_2 \cdot [C]_0} \quad (8)$$

$$y = 0.587 + 0.061(\pm 0.002) \cdot x(\text{“fast” reaction for the β-CD-assisted Cy3G solution}) \quad r = 0.999, r^2 = 0.998, F = 642.2, p < 0.03, s = 0.03 \quad (9)$$

$$y = 0.694 + 0.058(\pm 0.000) \cdot x(\text{“slow” reaction for theβ-CD-assisted Cy3G solution}) \quad r = 0.99996, r^2 = 0.99991, F = 69712, p < 0.00001, s = 0.03 \quad (10)$$

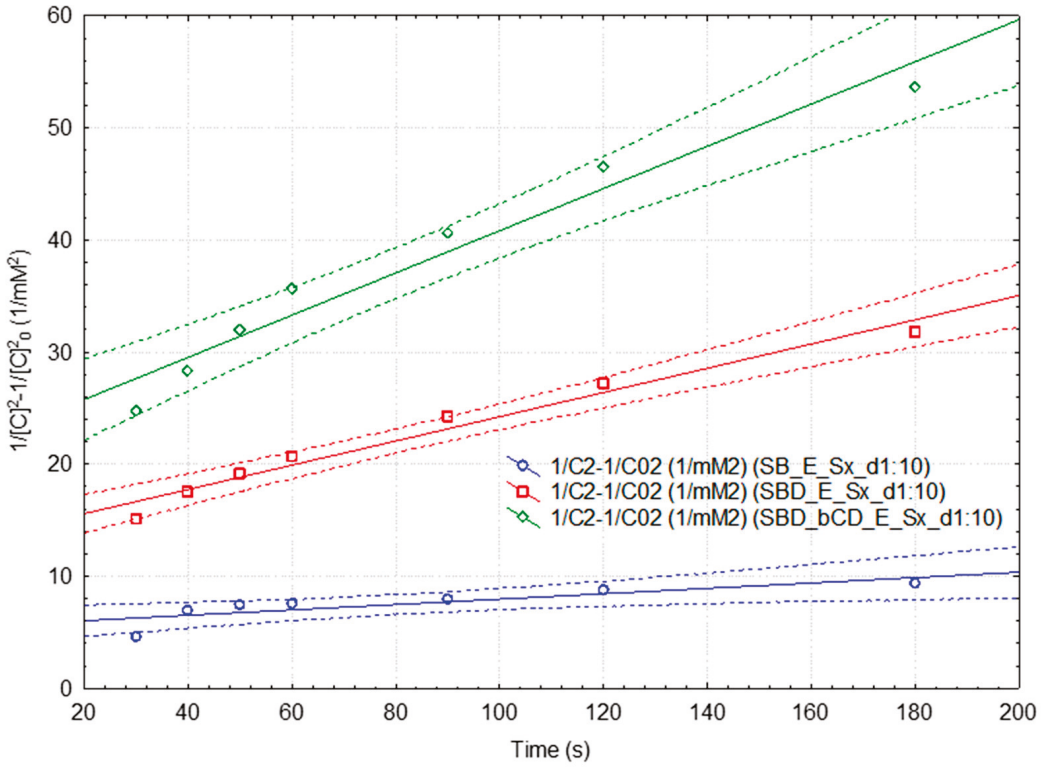


Figure 6. Integrated rate law representation for the third (pseudo-)order kinetic model for the “slow” DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh and dehydrated strawberries (without or with β-cyclodextrin as additive; 1:10 diluted extracts). Mean values and 95% confidence interval (dotted lines) were used.

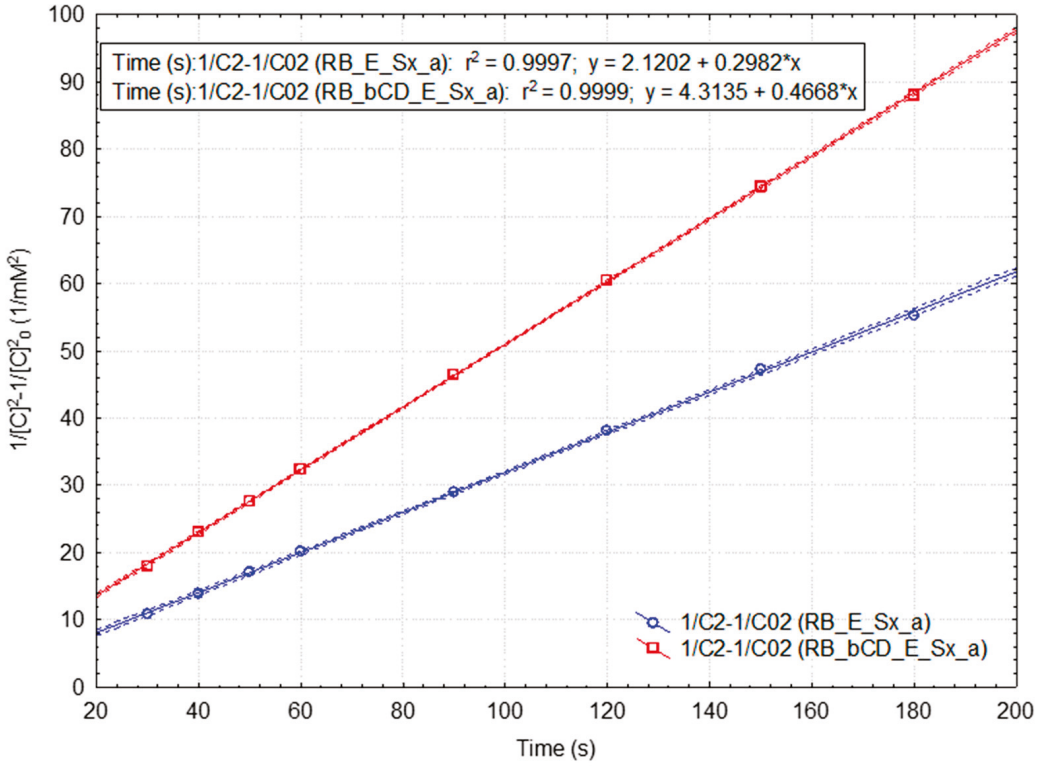


Figure 7. Integrated rate law representation for the third (pseudo-)order kinetic model for the "slow" DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh raspberries (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). Mean values and 95% confidence interval (dotted lines) were used.

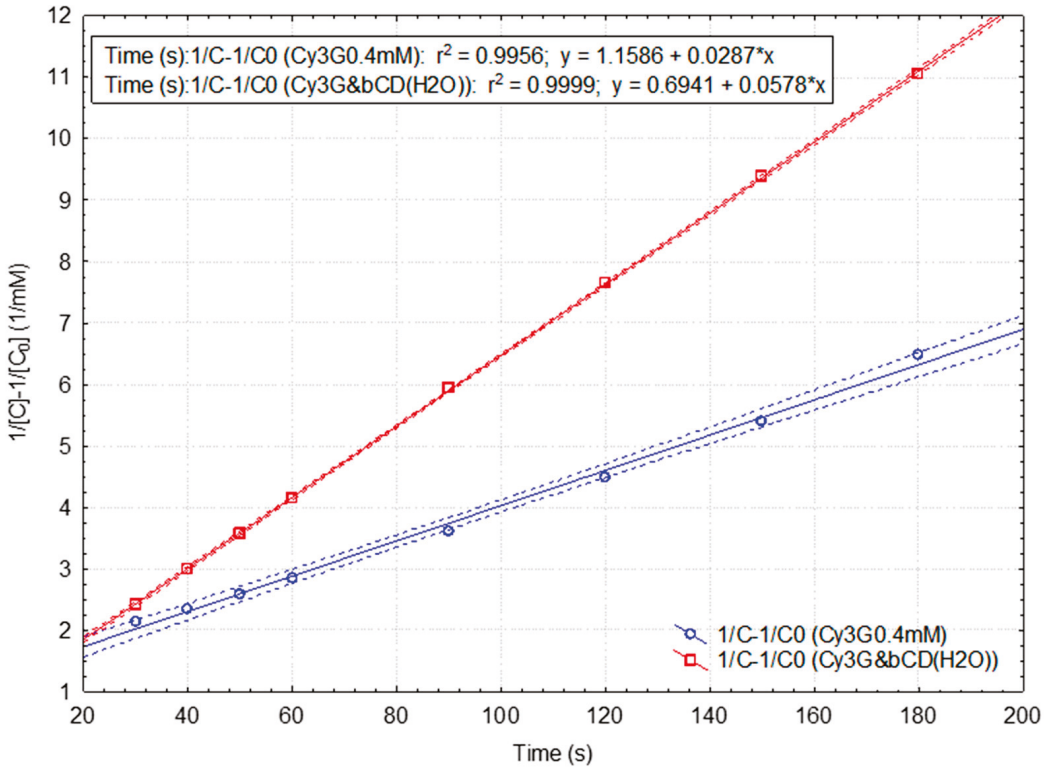


Figure 8. Integrated rate law representation for the second (pseudo-)order kinetic model for the "slow" DPPH· reaction with the standard 0.4 mM cyanidin 3-*O*-glucoside (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). Mean values and 95% confidence interval (dotted lines) were used.

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3. Discussion

This study focuses on the influence of natural β-CD during both convective air-dehydration of berries and reaction of antioxidants in berry extracts with free radicals, which has been investigated and is reported here for the first time. There were statistically significant differences in terms of antioxidant activity and radical reaction kinetics.

3.1. Identification and Quantification of Anthocyanins

Eleven anthocyanins and anthocyanidins were separated using HPLC in SB extracts. The identification and quantification was performed for the main antioxidant compounds in SB, i.e., Cy3G and Cy. Plg3G and total anthocyanin and anthocyanidin content were also estimated as Cy3G. Cy3G content in fresh SB was 0.32 ± 0.08 mg/100 g fw, but Cy was identified at only 0.017 ± 0.003 mg/100 g fw. However, Plg3G was the most concentrated in fresh SB, being identified at 2.46 ± 0.38 mg/100 g fw, more than a half of the total anthocyanin and anthocyanidin content (4.28 ± 0.56 mg Cy3G/100 g fw). The identification of the main anthocyanins in berry extracts was also supported by the statistically significant correlation of the retention time (RT) parameter in RP-HPLC analysis with the logarithm of the octanol/water partition coefficient, LogP, which is strongly related with the partition of the antioxidant compounds between the hydrophobic stationary phase (Nucleosil 100 C18 column based on octadecylsilane structure) and the hydrophilic mobile phase (acidified acetonitrile–water mixture) [62,63]. LogP values for anthocyanins were calculated using the atomic contributions implemented in the QSAR Properties module in HyperChem 7.52 (HyperCube, Inc., Gainesville, FL, USA). Further information can be found in the Figures S21–S23 in the Supplementary Materials file. Thus, for the identified anthocyanins, i.e., Cy3G, Plg3G, and Cy, the RT versus logP correlation is given below (Equation (11)).

$$\begin{gathered} RT_{HPLC}(min) = 10.444(\pm 0.564)\cdot LogP \\ r = 0.997, r^2 = 0.994, F = 342.4, p < 0.003, s = 1.58 \end{gathered} \quad (11)$$

SB composition is in line with other research on the fresh berries. However, there are large differences in both the specific anthocyanin content and total anthocyanin content. De-Souza and collaborators determined the total anthocyanin content at 16.03 mg Cy3G/100 g fw [25], while Chaves et al. quantified the total anthocyanins in fresh SB at 15.67–23.46 mg Plg3G/100 g [19]. Similar values of 15.45 and 19.95 mg/100 g fw were obtained after various pretreatments of the plants (e.g., Lemon verbena bio extract applied to SB plants at 200–600 mg/L) [64,65]. Higher total anthocyanin contents of 38–176 mg/100 g and 98 mg Cy3G/100 g fw were obtained by Dzhanfezova et al. and Jiang et al. [20,42]. On the other hand, Hussain et al. determined lower content of anthocyanins in fresh SB at only 3.17 mg/100 g fw [3]. Plg3G was determined in fresh SB at the highest content (up to 170 mg/100 g fw) by comparison with the other anthocyanins. However, the Plg3G content was quantified at 0.31–59.86 [21], 4.157–6.235 [18], 14.05–22.30 [19], 18.02 [65], 25.066 [52], and 30–170 mg/100 g [20]. In all cases, Cy3G was less concentrated, with a range of 0.68–28 mg/100 g in fresh SB (0.68 [52], 0.8–28 [20], 0.83–1.99 [19], 1.39 [65], 1.988–2.774 [18], and up to 6.01 mg/100 g [21]).

Fresh RB had higher total anthocyanin and anthocyanidin content of 73.18 ± 12.39 mg Cy3G/100 g fw, while the specific Cy3G and Cy were identified at 14.62 ± 2.64 and 0.64 ± 0.07 mg/100 g fw, respectively. These values are in agreement with the other results obtained by Wang et al. for RB of 6.39 ± 0.02 mg Cy3G/100 g fw, among other anthocyanins such as cyanidin 3-sophoroside or cyanidin 3-(6′-citryl)-sophoroside [28]. However, the total anthocyanin content determined in other studies vary in a wide range of 14.69–98.91 mg/100 g, depending on the RB growing and process-

ing conditions [25,26,28]. For example, these values increased after using biostimulants at 10.71–24.85 mg Plg3G/100 g in the first year and to 35.12–58.87 mg Plg3G/100 g for the second year of treatment [26]. On the other hand, the total anthocyanin content of the RB subjected to subcritical water extraction at 7 MPa and 130 °C for 90 min reached 98.91 ± 0.33 mg/100 g [28].

3.2. Dehydration of Strawberry Fruits

The dehydration and drying of fruits induces the partial degradation of antioxidant compounds, especially if the air–oxygen and higher temperature are used like in the case of classical convective air-dehydration used in this study, which reduced the moisture content of SB by 91%. The total anthocyanin content, as well as the content of the main anthocyanins in β-CD-assisted dehydrated SB was slightly higher than in the fresh SB (4.30 mg Cy3G/100 g for the total anthocyanin and anthocyanidin content). On the other hand, the anthocyanin compounds were identified at lower contents, but their aglycones were more concentrated in the β-CD-assisted dehydrated SB. For example, only 1.64 and 0.15 mg/100 g were determined for Plg3G and Cy3G, but the aglycone Cy was more concentrated (0.033 mg/100 g). Unfortunately, the dehydration without β-CD addition provided products with very low content of anthocyanins and anthocyanidins, which most probably were degraded to other compounds during the longer thermal process. There are various studies on the degradation of anthocyanins as pure compounds or in fruit extracts. Cy3G content is reduced at a third from the starting concentration in ethanol solution subjected to 95 °C for 30 min, especially under pressure [66]. The PEF (Pulsed Electric Field) processing also affect the stability of Cy3G [67]. Anthocyanins in blueberry extracts were significantly degraded under heat and acid treatments. Almost no Cy3G, as well as malvidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, malvidin 3-*O*-galactoside, petunidin 3-*O*-glucoside, petunidin 3-*O*-galactoside, delphinidin 3-*O*-glucoside, cyanidin 3-*O*-galactoside, delphinidin 3-*O*-galactoside, or cyanidin 3-*O*-arabinoside, were identified after the treatment of TFA (trifluoroacetic acid) acidified blueberry extract at 95 °C for 90 min [68]. The anthocyanin content decreased very fast in SB concentrates subjected to heating at 95 °C and pH 3.5 for 6 h. The total anthocyanin content decreased at only 40.39 mg/L from a starting value of 171.35 mg/L. The half-time for SB was the highest (1.95 h) in comparison with elderberry and black carrot concentrate cases [69]. Total anthocyanin content was slightly modified after spray-drying of SB at 8.04 mg/100 g (from the starting content of 3.17 mg/100 g in fresh SB) [3]. The total anthocyanin content was not significantly modified after various pretreatments (freeze–thaw, ultrasound, freeze–ultrasound) in infrared combined hot-air-impingement drying of SB slices, from the 98 to 108–119 mg Cy3G/100 g, related to the dry basis [42].

Many anthocyanin degradation compounds are generated under heat and oxygen, although some of them can retain the antioxidant properties. The first step is deglycosylation (hydrolysis) to the corresponding aglycone (e.g., Cy), followed by various cleavages providing smaller compounds such as phloroglucinaldehyde or protocatechuic acid [67,69]. The content of the degradation antioxidant compounds significantly increased to 11.89 μg/mL for 4-hydroxybenzoic acid and 4.89 μg/mL for phloroglucinaldehyde in the SB concentrate subjected to 95 °C and pH 3.5 for 4 h. These degradation compounds were not identified in the fresh products [69].

3.3. Antioxidant Activity of Berry Extracts

Antioxidant activity is one of the most important properties of berries, achieved through the radical scavenging capacity of the contained polyphenols: anthocyanins, anthocyanidins, flavonoids, hydroxycinnamic acids, and ellagitannins. However, these compounds are less

stable, especially during the thermal processing or in the presence of air [22,23,70,71]. Consequently, the use of β-CD as natural additive for convective air-dehydration of berry slices, as well as during the radical scavenging reactions of antioxidant compounds contained by berry extracts, was focused on the protection (enhancing stability) of antioxidant compounds and the modulation of their reactions with free radicals [72,73]. These applications of β-CD were studied for the first time and the results are very promising.

In all studied cases, β-CD addition enhanced the overall antioxidant activity, as revealed by the actual and final RSA values. This behavior was easily observed for the diluted extracts obtained from the β-CD-assisted dehydrated SB, by comparison with the not-assisted samples or the raw RB extracts. The RSA values were 3.5 times higher for the β-CD-assisted dehydrated samples and these differences are slightly lower for the β-CD-assisted reaction of free radicals with the antioxidant compounds in RB extracts, but statistically significant ($p < 0.05$). At the final monitoring, the RSA values for the β-CD-assisted reaction were 6% higher in comparison with the not-assisted reaction. However, the low solubility of β-CD in ethanol determined the use of ethanol–water solution at high dilution for maintaining the complete solubilization of both β-CD and its complexes with antioxidant compounds from the various classes, reaction products, and even free radicals.

The enhancement of the antioxidant activity by the β-CD is due to the efficient interaction of the more hydrophobic and geometrically compatible antioxidant moiety with the hydrophobic cavity of β-CD. Further, the hydrophilic moieties that contain phenolic groups are exposed to the solution (including by retention of these OH groups at the surface of the complex through H-bonding). These orientations of the antioxidant compounds enhanced the interaction with the free radicals, which mainly remains in the solution. DPPH· has a relatively rigid and bulk-like structure that is not compatible with the β-CD cavity (see Figures S24 and S25 in the Supplementary Materials file). This can also explain the differences between the RSA values, which are higher for the standard Cy3G solution analyzed in the presence of β-CD, in comparison with the not-assisted sample case. Moreover, the stabilization of the antioxidant compound during analysis can provide better reproducibility for this reaction. Significantly lower SD values were obtained for β-CD-assisted spectrophotometric reaction monitoring. However, further studies are needed for proving this observation.

The antioxidant activity of SB and RB extracts was determined in various studies for fresh or processed fruits. The most used method was the DPPH· assay, but $ABTS^{+}\cdot$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation), FRAP (ferric reducing antioxidant power), or TEAC (trolox equivalent antioxidant capacity) were also applied. Up to now, no studies regarding the modulation of the antioxidant activity by CDs were reported. However, the overall antioxidant activity of SB and RB varies in wide ranges, but the processing (thermal and/or oxidative) significantly decreases the antioxidant activity related parameter values, as was discussed in the present study for the dehydration process. Relevant published studies on antioxidant activity of both fresh and processed berries are discussed below.

Fresh SB and RB were studied for their antioxidant activity using DPPH·, $ABTS^{+}\cdot$, and β-carotene assays. The results revealed values of 3779 and 4961 g fw/g DPPH·, 7.87 and 6.27 μmol TE/g fw, respectively, and 67.13 and 75.19% protection [25]. DPPH· and FRAP assays were also applied for 14 SB cultivars that reveal high antioxidant activity during the two years of monitoring. The highest values of 9.1–11 mmol TE/g fw, respectively, 45–50 mmol Fe^{2+}/g fw, were obtained for wild SB (*Fragaria vesca* L.). By comparison, DPPH· and FRAP assays reveal values in the ranges of 3.1–5.6 mmol TE/g fw and 13.8–30 mmol Fe^{2+}/g fw for the *Fragaria* × *ananassa* Duch. cultivars [20]. Mandrave et al. determined the antioxidant activity of other two SB "Sweet Charlie" and "Camarosa" cultivars at four

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different fruit developmental stages. EC_{50} values for the DPPH· and FRAP assays were in the ranges of 9.71–130.4 mg/mL and 57.11–219.5 μg for the 0.2% acetic acid extracts, respectively, and 39.01–296.85 mg/mL and 13.90–202.03 μg for the ethanol extracts [74]. In another study on the 90 SB (*Fragaria* × *ananassa* Duch.) cultivars, the DPPH· and $ABTS^{+}$· assays provide values of 751.57–765.06 and 1531.17–1637.96 μmol TE/100 g fw for two years of monitoring [21]. Applying nano-fertilizers during the growing of berry plants influences the antioxidant activity of the berries, as was determined by the DPPH· scavenging activity for SB. The best result of 93.88% was obtained for a combined application into soil of a hybrid nanocomposite and a nano-zeolite fertilizer [75].

A study on the variation in the antioxidant activity (determined by TEAC assay) during heating of SB pigment solutions at 95 °C and pH 3.5 for 4 h revealed decreases in the TEAC values from 185.38 to 138.00 mg vitamin C equivalent/L, which means a reduced antioxidant activity by 25% [69]. On the other hand, high-intensity ultrasound treatment of the SB juice increased the antioxidant activity by 38%, as was determined using the DPPH· method. The DPPH· inhibition value increased from 31.5% to 43.5% for the ultrasound-treated samples (20 kHz, 400 W, and 12 min) [76].

Antioxidant activity of fresh red and black RB at various development stages was evaluated using FRAP, ascorbic acid (ASA), and GSH assays. Generally, the red RB had a slight increase or not-significant antioxidant activity during the developing stages. For red RBs, there were four stages of development, starting from the small immature green fruits to fully ripe dark red fruits easily detached from the crown. On the other hand, for black RBs, there were five stages of development from the small immature green fruits to fully ripe purple fruits. The black RB had a significant increase in the antioxidant activity parameters values at the fifth stage of maturity (FRAP 12 μmol/g, ASA 16 mg/g fw, and GSH 12.4 mg/g fw) [77]. For another seven RB cultivars, the antioxidant activity was determined by DPPH· and ORAC assays with values of 305–350 and 136.7–205.2 μmol TE/g dw (dry weight), respectively [78]. The use of microbial biostimulants (*Bacillus subtilis* (Ehrenberg) Cohn and/or *Paenibacillus* Ash et al., 1994 species) increased the overall antioxidant activity of RB by 16–20%. For example, the DPPH· value increases from 55.02% to 62.4% for "Delniwa" cultivar if a biostimulant based on 6.25 mL liquid preparation containing *B. subtilis* (B4/19-AF75AB2) and *B. subtilis* (B7/19-Sp115AD), 6.25 mL liquid preparation containing *B. subtilis* (B7/19-Sp115AD) and *Paenibacillus* sp. (B13/19-Sp116AC), 37.5 mL water and 10 g powder preparation containing *B. subtilis* (B4/19-AF75AB2) and *Bacillus* sp. (B6/19-AF75BC) was applied on soil 2–3 times during the growing season [26]. In another study, the anthocyanins separated from RB by subcritical water extraction (at 100 μg/mL) had the highest antioxidant activity (83.77% by DPPH· assay and 66.7% by $ABTS^{+}$· assay) in comparison with the hot water or methanol extracts at the same anthocyanin content [28].

Dehydration and drying significantly influenced the antioxidant activity of berries, which strongly depend on the technique used. Osmotic dehydration pretreatment, followed by freeze-drying of SB, decreased the antioxidant activity by DPPH· technique from 85.24% for the fresh berries to only 42.94% for the freeze-dried samples, which is in line with our results on convective air-dehydration [79]. On the other hand, infrared combined hot air impingement drying of SB slices provide antioxidant activities in the range of 62.34–62.69% if the freeze–thaw, ultrasound, or freeze–ultrasound pretreatments were used [42]. Santos et al. also used ultrasound and ethanol pretreatments for enhancing the antioxidant activity of SB slices subjected to convective air-drying. The best results were obtained for ultrasound and 50% ethanol pretreatment. The DPPH·, $ABTS^{+}$·, and FRAP values decreases by 10.32, 13.78, and 6.54%, respectively [80].

CDs were only used for obtaining biofilms or other materials designed for preservation of fresh berries. Antioxidant activity of such preserved fruits were determined using the

methods presented above. The DPPH· assay revealed values of ~48% for SB preserved with a biofilm based on chitosan/polyvinyl alcohol loading β-acids/β-CD inclusion complex in comparison with the control sample of 40% after three days of storage [45]. Enhancement of the antioxidant activity and stability was also obtained if a hydrogel based on κ-carrageenan and carvacrol/hydroxypropyl-β-CD composite, nerolidol/hydroxypropyl-β-CD inclusion complex nanofibers, or a film composite based on Pickering nanoemulsion loaded with eugenol/β-CD and konjac glucomannan were applied to SB fruits [46,48,51].

3.4. Kinetic Studies

Both the DPPH· and ABTS+· methods were widely used for the evaluation of the antioxidant activity of berry extracts. One of the scope of this study was the evaluation of the reaction of a free radical with antioxidant compounds in berry extracts in the absence or presence of β-CD. In this regard, the reaction kinetics ("fast" and "slow") were evaluated. By far, DPPH· reaction kinetics is the most studied, especially for the reaction with antioxidant compounds [55,58–60,81–85]. The $DPPH_2$ with absorbance < 450 nm was obtained as the reaction product. On the other hand, reaction kinetics involving $ABTS^{+}$· radical cation is less studied. Only a few studies regarding the reaction kinetics between $ABTS^{+}$· and flavonoids or other antioxidants were found in the literature [86–89]. Another problem is related to the formation of two species, i.e., $ABTS^{+}$ and $ABTS^{2+}$, as the products of the reaction with antioxidant compounds [86].

DPPH· kinetics during the reaction with antioxidant compounds in berry extracts in the presence of β-CD can provide further information on the stability and behavior of anthocyanins as free radical scavengers. β-CD comes from the assisted dehydration of SB, when β-CD is partially transferred during the extraction process or is used as additive during the DPPH· reaction with antioxidants from RB extracts. As previously presented for both "fast" and "slow" reactions (classification of the reaction types according to [61]), the best fits and statistical parameters were obtained for the higher-(pseudo-)order kinetic models (only related to DPPH· concentration), i.e., third- and fourth-(pseudo-)order kinetic models. On the other hand, pure Cy3G reveals lower-(pseudo-)order kinetic models. These differences on the kinetic behavior can be explained by the very complex antioxidant system/mixture, not only as specific polyphenols, but also as different classes of compounds. However, the DPPH· reaction behavior is similar for berry extracts and pure Cy3G assisted or not by β-CD. This is due to the favorable interactions of antioxidant compounds with β-CD cavity, regardless the chemical class of antioxidants [72,90–101]. According to these DPPH· kinetic models, the rate constant for the "fast" reaction with antioxidants from β-CD-assisted dehydrated SB was three to five times higher than the corresponding rate constant for the fresh SB extract. This behavior was also observed for the "slow" reaction, but the differences between rate constant values are even higher for the case of third-(pseudo-)order kinetic model. Consequently, the DPPH· reaction in the presence of β-CD is faster. These aspects were also observed during the DPPH· reaction with antioxidant compounds in RB extracts in the presence of β-CD, but the differences are less significant, such as for the pure Cy3G case. However, no kinetic studies were published for the DPPH· reaction with antioxidant compounds in the presence of CDs. There are some kinetic studies on the DPPH· reaction with flavonols, flavanols and hydroxycinnamic acids (rate constants for the first-order kinetics of 400–5000 $L \cdot mol^{-1} \cdot s^{-1}$) [59], flavonol quercetin (first-order kinetics) [60], flavanone naringenin in micellar media [85], or curcumin (rate constants of the first-order kinetics of $1.8 \cdot 10^4$ $L \cdot mol^{-1} \cdot s^{-1}$) [82]. Other kinetic studies are related to the anthocyanin degradation processes or hydrolysis reactions under various conditions, even as pure antioxidant compounds or in fruit extracts [68,102–106].

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All the mentioned examples focus on bimolecular reactions and were proven to follow lower-order kinetic models, such as in our study on the reaction of DPPH· with pure Cy3G. However, DPPH· kinetic models for complex reactions with antioxidants in fruit extracts are more difficult to evaluate. This is the case of "fast" and "slow" kinetics with antioxidants in citrus juices [58]. Other DPPH· kinetic studies were performed on the "extremely fast" reaction region (e.g., up to 10 s), as well as the "fast" and "slow"/"slower" regions, for gallic acid, methyl gallate, ascorbic acid, catechin, epicatechin, quercetin, rutin, tannic, ellagic, and syringic acids, rutin in micellar nanostructures, as well as for antioxidants from various fruit juices such as red plum or SB [55,61,81].

4. Materials and Methods

4.1. Plant Materials and Chemicals

Fresh SBs (*Fragaria* × *ananassa* Duch. var. Albion) were purchased from the local producer (batch number 1905, IamaAgri Strawberry Factory, Arad, Arad county, Romania; growing location: Peciu Nou, Timiş county, Romania; growing coordinates and conditions: 45°36′23″ N and 21°3′28″ E, natural pollination and biological pest control; harvesting period: May 2022). Fresh fruits were stored at 4 °C in the original package for maximum two days until the convective drying experiments.

Fresh European RBs (*Rubus idaeus* L.) were purchased as frozen whole fruits from a producer from Transylvania (batch number RO.10.VI.22, S.C. Transylvania Royal Food S.R.L., Târnăveni, Mureş county, Romania; growing coordinates: 46°19′47″ N and 24°16′12″ E; harvesting period: June 2022). The fruits were stored at −18 °C until extraction process.

Ethanol (96%, *v*/*v*, ChemiCal Co, Bucharest, Romania) was used for both extraction and spectrophotometric analysis. β-Cyclodextrin hydrate, β-CD (Kleptose®), was purchased from Roquette Freres S.A. (Lestrem, France) and had a purity > 98% (max. 0.25% α-CD, max. 0.25% γ-CD, max. 0.5% other impurities, max. 14% water content). Cyanidin 3-*O*-glucoside chloride, Cy3G (from *Rubus fruticosus*, purity > 97% by HPLC) and cyanidin chloride, Cy (from *Aronia melanocarpa*, purity > 97%), were purchased from PhytoPlan® Diehm & Neuberger GmbH (Heidelberg, Germany). Acetonitrile (HPLC grade, Honeywell | Riedel-de Haën, Seelze, Germany) and trifluoroacetic acid ("for analysis" grade, Carlo Erba Reagents S.A.S., Val-de-Reuil, France) were used for chromatographic analysis. 2,2-Diphenyl-1-picrylhydrazyl (DPPH·) for radical scavenging activity and reaction kinetics had a purity > 99.5% and was obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

4.2. Dehydration of Fruits in the Presence or Absence of β-Cyclodextrin

Dehydration was applied for fresh SB slices using a SilverCrest SDA 350 A2 (350 W) convective air-dehydrator (HOYER Handel GmbH, Hamburg, Germany). Fresh fruits were washed with distilled water, blotted with filter paper for removing the remaining water, and sliced along the longitudinal axis at a thickness of ~3–4 mm. Dehydration was performed using two methods.

Classical convective air-dehydration. Around 133.6 ± 1.6 g of sliced fruits were weighted at the starting of dehydration, placed on the dehydration trays at distances of about 1 cm between slices (Figure 9). The dehydration time and temperature were set at 12 h and 70 °C, respectively (according to the producer for these types of samples). After the dehydration process, the samples were weighted again and stored at 4 °C in a sealed glass flask until extraction.

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

Figure 9. Strawberry dehydration process. (**a**) Overview of the convective air-dehydrator; (**b**) parameter setting (e.g., temperature) for the strawberry dehydration.

β-CD-assisted convective air-dehydration. β-CD-assisted convective air-dehydration was applied for the first time using β-CD as natural additive for enhancing the water diffusion from the inner parts of the samples as well as for protection of the antioxidant compounds against degradation (in the presence of air/oxygen and high temperature). The same amount of sliced strawberries (133.7 g) was uniformly covered with powdery β-CD hydrate (1.88 g) and immediately subjected to air-dehydration in the same conditions (70 °C for 12 h). After weighting, the dehydrated samples were stored at 4 °C until extraction.

4.3. Extraction of Antioxidants from Fresh and Dehydrated Fruits

Fresh and dehydrated fruits were subjected to the classical semi-continuous solid–liquid extraction according to the literature for the separation of anthocyanins and other polyphenols from fruits using ethanol as "green" solvent [107,108]. Slight modifications were applied to these methods from the literature. Thus, 250 mL or 100 mL Soxhlet extractors were used for the fresh and dehydrated samples, respectively. Soxhlet extractors were equipped with reflux condensers. Approximately 25 g of fresh fruits or 12–13 g of dehydrated fruits were finely grounded in a mortar and transferred into the extractor's cellulose cartridge. In total, 300 mL of 96% ethanol was added to the 500 mL round-bottom flask for the fresh samples. In total, 150 mL of 96% ethanol was used for dehydrated samples. The flasks were heated on water baths and the samples were extracted for at least five times (until no color was observed in the final extract of the multiple extraction process and the HPLC analysis of the last extract did not reveal any significant peaks for anthocyanins). After cooling, all the final extracts were completed with ethanol at a final volume of 300 and 150 mL for the fresh and dehydrated samples, respectively. SB and RB extracts were hermetically sealed in glass flasks until the UV-Vis spectrophotometry and HPLC analyses (they were stored at 4 °C up to two days if necessary).

4.4. HPLC Analysis of the Main Anthocyanins from Fruits

HPLC analysis was performed for identification and quantification of the main anthocyanin derivatives in both SB and RB extracts—Cy3G, as well as its aglycone, Cy—and correlation with the antioxidant and kinetic data. Analytical HPLC/UV-Vis Jasco (Jasco Corporation, Tokyo, Japan) equipment was used. The HPLC was equipped with a PU-2080 Plus

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Intelligent Pump, LG-2080-04 gradient unit, LG-2080-54 4-Line Degasser, UV-2070Plus Intelligent Detector, and LC-Net II/ADC interface (all from Jasco Corporation, Tokyo, Japan). The HPLC conditions were set as follows: Nucleosil 100 C18 column (250 mm × 4 mm, 5 μm), wavelength for anthocyanin detection 525 nm, flow 1 mL/min, room temperature; the mobile phase was composed of the solvent A (0.4% TFA in distilled water) and solvent B (0.4% TFA in acetonitrile); the gradient used for analysis was 15% B (0–6 min), 15–22% B (6–20 min), 22–35% B (20–35 min), 35–15% B (35–40 min), and 15% B for 5 min [109]. The sample volume was set for 20 μL. Acquisition and handling of the data were performed using the ChromPass Chromatography Data System version 1.7.403.1 from the same manufacturer. Limit of detection (LOD) and limit of quantitation (LOQ) values were determined according to the information provided by the HPLC equipment's instructions and were computed using the same ChromPass Chromatography Data System version 1.7.403.1 software. Thus, five chromatograms of every standard anthocyanin compounds (i.e., Cy3G and Cy) were selected and the "noise" values in the range of ~5 min (close to the corresponding peak, but without any other secondary peaks) were determined. The LOD and LOQ values were calculated as 3·"noise" and 5·"noise", respectively. The mean LOD and LOQ values for Cy3G and Cy were 2.54 and 4.23 μg/mL, respectively, 1.31 and 2.18 μg/mL (n = 5). RT versus LogP correlation confirmed the identification of the anthocyanin compounds in berry extracts. LogP was determined using the QSAR Properties program from the HyperChem version 7.1 software package and was based on the atomic parameters, according to Ghose et al. and Viswanadhan et al. [110,111].

4.5. Radical Scavenging Activity of Fruit Extracts

The antioxidant activity of the berry extracts was determined using the DPPH· method according to the literature [3,65,80], with slight modifications presented below for the not-assisted and β-CD-assisted cases. The behavior (reaction) of the 0.4 mM Cy3G ethanol solution or 1:10 diluted berry extracts in the presence of 1 mM DPPH· ethanol solution was evaluated using a Camspec M501 Single-Beam spectrophotometer (Camspec Ltd., Cambridge, UK). The acquisition and handling of the UV-Vis data were performed using the UV-Vis Analyst version 4.67 software from Camspec Ltd. (Cambridge, UK). Thus, a mixture of standard solution (0.4 mM Cy3G) or 1:10 diluted berry extract and 1 mM DPPH· solution and solvent (ethanol) at a volume ratio of 1:1:4 was monitored for 15 min at 517 nm. Possible absorbance interferences for DPPH· and anthocyanins can appear. Consequently, the absorbance of blank samples (0.4 mM Cy3G or 1:10 diluted berry extract and ethanol at a volume ratio of 1:5) was subtracted. However, the absorbance of the blank samples was less than 15% from the absorbance of the sample mixtures containing DPPH· solution at the starting of monitoring. The spectrophotometric monitoring in the presence of β-CD solution was performed in a similar way. The sample (Cy3G or diluted RB extract) was mixed with aqueous 1 mM β-CD solution, 1 mM DPPH· solution, and ethanol at a volume ratio of 1:1:1:3, followed by spectrophotometric monitoring for 15 min at 517 nm.

The antioxidant activity was evaluated by determining the actual RSA (radical scavenging activity) values during the monitoring (Equation (12)).

$$RSA_t(\%) = \left[1 - \frac{Abs_t}{Abs_0}\right] \cdot 100 \quad (12)$$

where RSA_t (%) stands for the radical scavenging activity at the time t and Abs_t and Abs_0 stand for the absorbance of the mixture at the time t and t = 0 s, respectively. A high RSA value at the final of monitoring period means high antioxidant activity, while the fast or slow changes in RSA values can provide information on how the antioxidant compounds

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in berry extracts can have a prolonged activity. These aspects were evaluated using DPPH· reaction kinetics (see below).

4.6. DPPH· Reaction Kinetics in the Presence of Fruit Extracts and Standard Anthocyanins

Kinetic parameters related to the reaction between DPPH· and various antioxidant compounds in berry extracts can provide information on the behavior during the radical scavenging in vivo, as well as the influence of β-CD during the reaction. The actual concentration of the DPPH· radical during the reaction can be easily monitored using spectrophotometry using the "Time scan measurement" module (the interference of anthocyanins that absorb at 517 nm was appropriately considered and the corresponding blank absorbance was subtracted; see Figure S3 in the Supplementary Materials file). In this regard, the DPPH· calibration curve in the range of 0.01 to 0.20 mM was obtained (DPPH· Concentration (in mM) versus Absorbance (at 517 nm); Equation (13), Figures S4 and S5 in the Supplementary Materials file).

$$\begin{gathered} \mathrm{Conc_{DPPH\cdot}(mM)} = 0.1044(\pm 0.0004)\cdot \mathrm{Abs_{(@517nm)}} \\ \mathrm{n} = 7, \mathrm{r} = 0.99996, \mathrm{r}^2 = 0.99992, \mathrm{F} = 70829, \mathrm{p} < 10^{-5}, \mathrm{s} = 0.0011 \end{gathered} \quad (13)$$

The variation in the DPPH· concentration during the 15 min of monitoring reveals the complex reactions of this free radical with antioxidant compounds in berry extracts, such as Cy3G. Because the reaction is "fast" up to 30 s then becomes "slower", three time reaction ranges were considered: 0–30 s for the "fast" reaction, 30–180 s for the "slow" reaction, and 180–900 s for the "prolonged" reaction (not studied for the kinetics point of view). Five kinetic models related to the variation in the DPPH· concentration were considered, i.e., zeroth- to fourth-(pseudo-)order kinetics (Equation (14) for the general rate law). The linear plot of the integrated rate law (integrated rate law for nth-order reaction, Equation (15)) allows us to determine the reaction rate constant, k_n, and, further, the half-time, $t_{1/2}$ (Equation (16)), of the DPPH· for the selected time range.

$$-\frac{d[\mathrm{C}]}{dt} = \mathrm{k}\cdot[\mathrm{C}]^n \quad (14)$$

$$\begin{gathered} \frac{1}{[\mathrm{C}]^{-\mathrm{g}}} - \frac{1}{[\mathrm{C}]_0^{-\mathrm{g}}} = -\mathrm{g}\cdot\mathrm{k}\cdot\mathrm{t}, \text{ for } \mathrm{n} \neq 1, \mathrm{g} = 1-\mathrm{n}, \langle \mathrm{k} \rangle = \frac{1}{\mathrm{mM}^{-\mathrm{g}}\cdot\mathrm{s}} \\ \ln\left(\frac{[\mathrm{C}]}{[\mathrm{C}]_0}\right) = -\mathrm{k}\cdot\mathrm{t}, \text{ for } \mathrm{n} = 1 \end{gathered} \quad (15)$$

$$\mathrm{t}_{1/2} = \frac{1-2^{-\mathrm{g}}}{\mathrm{g}}\cdot\frac{1}{\mathrm{k}\cdot[\mathrm{C}]_0^{-\mathrm{g}}}, \text{ for } \mathrm{n} \neq 1, \mathrm{g} = 1-\mathrm{n}, \mathrm{t}_{1/2} = \frac{\ln(2)}{\mathrm{k}}, \text{for } \mathrm{n} = 1, \langle \mathrm{t}_{1/2} \rangle = \mathrm{s} \quad (16)$$

4.7. Statistical Analysis

Extractions and spectrophotometric determinations were made at least as duplicates. Results are presented as mean ± standard deviation, SD (unless otherwise stated), which were determined using the Basic Statistics module in the Statistica version 7.1 software package (StatSoft Inc., Tulsa, OK, USA) and Microsoft® Excel® 2016 in Microsoft Office Professional Plus 2016 (Microsoft Corporation, Redmond, WA, USA). Linear regression analysis (calibration curves and integrated rate law linear regression) was performed using the Multiple Linear Regression module, while the non-linear regression was performed using the Generalized Nonlinear Models module in Statistica version 7.1 package. The quality of the regression analysis was evaluated using the statistical parameters for the equation (Pearson correlation coefficient, r, determination coefficient, r^2, adjusted determination coefficient, r^2_{adj}, Fisher and p values, and standard error of estimate, s) and for the raw correlation coefficients (standard error of the coefficient). On the other hand, the representation of the results

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for multiple determinations was performed using the Means with Error Plots module in the Statistica version 7.1 package, using the Whiskers graph type, Time as the grouping variable, and values as mean ± 0.95·standard error (vertical bars).

5. Conclusions

For the first time, the application of β-CD for enhancing the quality of the air-dehydrated berries was studied and, further, the modulation of the interactions between free radicals and antioxidants in berry extracts by β-CD was proved. In both cases, antioxidant activity of SB and RB extracts were significantly higher in the presence of β-CD. However, the best results for free radical reaction kinetics were obtained for higher-(pseudo-)order kinetic models (third- and fourth-(pseudo-)order, related to the free radical concentration). By comparison, pure anthocyanins provide good results for lower-(pseudo-)order kinetics, which is in line with other published kinetic studies. This difference can be due to the complexity of parallel and consecutive reactions involving antioxidants in extracts and free radicals, especially in the presence of β-CD that forms molecular inclusion compounds with these bioactive compounds. Finally, the protection capacity of β-CD against the degradation of antioxidants during the dehydration process of berries was proved. The technology can be extended to other fruits and scale up for obtaining high-quality fruit-based products.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/plants15010152/s1. Figure S1: Concentration (mg/mL) versus HPLC peak area (mV·min) calibration curve for the cyanidin 3-*O*-glucoside standard solutions in the range of 0.05–0.50 mg/mL; Figure S2: Concentration (mg/mL) versus HPLC peak area (mV·min) calibration curve for the cyanidin standard solutions in the range of 0.025–0.50 mg/mL; Figure S3: Comparison between the UV-Vis spectra (range of 400–700 nm) of the cyanidin 3-*O*-glucoside (Cy3G) standard solution (e.g., 0.06 mM—blue) and the standard DPPH· solutions (e.g., from bottom to top: 0.01, 0.02 and 0.16 mM, which is the working concentration at the start of the measurements—green). The Cy3G standard solution has a maximum absorbance in the range of the high diluted DPPH· solutions of 0.01 and 0.02 mM. However, the Cy3G working concentration at the start of the measurement is four times lower (0.015 mM). The spectra for the blank sample (ethanol) is in red; Figure S4: UV-Vis spectra (region 400–700 nm) of the DPPH· standard solutions (from bottom to top, concentrations from 0.01, 0.02, 0.04, 0.08, 0.12, 0.16 and 0.20 mM). The spectra for the blank sample (ethanol) is in red; Figure S5: Concentration (mM) versus Absorbance (@517 nm) calibration curve for the DPPH· standard solutions in the range of 0.01–0.20 mM; Figure S6: UV-Vis monitoring (at 517 nm) of the 0.4 mM cyanidin 3-*O*-glucoside and 1 mM DPPH· solutions in ethanol, at a volume ratio of 1:1:4 (duplicate samples; blank ethanol is in red); Figure S7: UV-Vis monitoring (at 517 nm) of the 0.4 mM cyanidin 3-*O*-glucoside, 1 mM DPPH· and 1 mM β-cyclodextrin (in water) solutions, mixed with ethanol at a volume ratio of 1:1:1:3 (duplicate samples; blank ethanol is in red); Figure S8: Superimposed Absorbance versus Time plots from the UV-Vis monitoring (at 517 nm) of the 0.4 mM cyanidin 3-*O*-glucoside and 1 mM DPPH· solutions in ethanol at 1:1:4 volume ratio (duplicate samples; blue colors) and for the 0.4 mM cyanidin 3-*O*-glucoside, 1 mM DPPH· and 1 mM β-cyclodextrin (in water) solutions, mixed with ethanol at a volume ratio of 1:1:1:3 (duplicate samples; green colors) (blank ethanol is in red, blank cyanidin 3-*O*-glucoside solution is in purple and blank DPPH· solution is in black); Figure S9: Antioxidant activity of the fresh strawberry extracts, expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min, as well as the logarithmic correlations (duplicate samples); Figure S10: Antioxidant activity of the fresh strawberry extracts (1:10 dilutions), expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min, as well as the logarithmic correlations (duplicate samples); Figure S11: Antioxidant activity of the extracts obtained from dehydrated strawberries, without (blue) and with β-cyclodextrin (red) as additive (1:10 dilutions). Antioxidant activity is expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min. Logarithmic

correlations are also provided; Figure S12: Antioxidant activity of the extracts obtained from fresh (blue) and dehydrated strawberries, without (red) and with β-cyclodextrin (green) as additive (1:10 dilutions). Antioxidant activity is expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min. Logarithmic correlations are also provided; Figure S13: Antioxidant activity of the extracts obtained from fresh raspberries, β-cyclodextrin assisted (green diamond) or not assisted (brown square) during the spectrophotometric monitoring (duplicate samples). Antioxidant activity is expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min. For multiple determinations, Whisker type plots as mean ± 0.95·standard error were used. Logarithmic correlations are also provided; Figure S14: Antioxidant activity of the standard 0.4 mM cyanidin 3-*O*-glucoside, β-cyclodextrin assisted (green diamond) or not assisted (brown square) during the spectrophotometric monitoring (duplicate samples). Antioxidant activity is expressed as the variation in the Radical Scavenging Activity (RSA, %) in Time (s) for 15 min. For multiple determinations, Whisker type plots as mean ± 0.95·standard error were used. Logarithmic correlations are also provided; Figure S15: Integrated rate law representation for the zeroth–fourth-order models for the "fast" DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh and dehydrated strawberries (without or with β-cyclodextrin as additive; 1:10 diluted extracts). For duplicate samples, Whisker type plots as mean ± 0.95·standard error were used; Figure S16: Integrated rate law representation for the zeroth–fourth-order models for the "slow" DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh and dehydrated strawberries (without or with β-cyclodextrin as additive; 1:10 diluted extracts). For duplicate samples, Whisker type plots as mean ± 0.95·standard error were used; Figure S17: Integrated rate law representation for the zeroth–fourth-order models for the "fast" DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh raspberries (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). For duplicate samples, Whisker type plots as mean ± 0.95·standard error were used; Figure S18: Integrated rate law representation for the zeroth–fourth-order models for the "slow" DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh raspberries (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). For duplicate samples, Whisker type plots as mean ± 0.95·standard error were used; Figure S19: Integrated rate law representation for the zeroth–fourth-order models for the "fast" DPPH· reaction with the standard 0.4 mM cyanidin 3-*O*-glucoside (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). For duplicate samples, Whisker type plots as mean ± 0.95·standard error were used; Figure S20: Integrated rate law representation for the zeroth–fourth-order models for the "slow" DPPH· reaction with the standard 0.4 mM cyanidin 3-*O*-glucoside (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). For duplicate samples, Whisker type plots as mean ± 0.95·standard error were used; Figure S21: Isosurface image for the most stable conformation of the cyanidin 3-*O*-glucoside (Cy3G). The total charge density was in the range of −0.3 to 0.3 (mapped function range) and is shown in the upper-left corner as a range legend; Figure S22: Isosurface image for the most stable conformation of the cyanidin (Cy). The total charge density was in the range of −0.3 to 0.3 (mapped function range) and is shown in the upper-left corner as a range legend; Figure S23: Isosurface image for the most stable conformation of the pelargonidin 3-*O*-glucoside (Plg3G). The total charge density was in the range of −0.3 to 0.3 (mapped function range) and is shown in the upper-left corner as a range legend; Figure S24: Optimized β-cyclodextrin/DPPH· complex, obtained with MM+ Molecular Mechanics Force Field module and Polak-Ribiere (Conjugate gradient) algorithm from the HyperChem 7.52 software package. Both β-CD and DPPH· were built and optimized separately as was presented above. The interaction starts with the DPPH· structure (both the Ph_2 and picryl sides) oriented to the secondary face of the β-CD, at a distance of ~8 Å between the gravity centers. DPPH· molecule is highlighted. The picryl moiety is only partial encapsulated into the β-CD cavity (at approximately 50%); Figure S25: Optimized β-cyclodextrin/cyanidin 3-*O*-glucoside complex, obtained with MM+ Molecular Mechanics Force Field module and Polak-Ribiere (Conjugate gradient) algorithm from the HyperChem 7.52 software package. Both β-CD and Cy3G were built and optimized separately as was presented above. The interaction starts with the Cy3G structure (benzopyrane moiety) oriented

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to the secondary face of the β-CD, at a distance of ~8 Å between the gravity centers. Cy3G molecule is highlighted. Benzo moiety is completely encapsulated. The 2-phenyl moiety (not encapsulated) has the 3- and 4-OH groups oriented to the solution, as well as the glucose moiety. This favors the reaction with the DPPH· radical from the solution; Table S1: Kinetic and statistic parameters corresponding to the zeroth–fourth-order models for the "fast" DPPH· reaction with antioxidant compounds from the 1:10 diluted extracts obtained from the fresh and dehydrated strawberries (without or with β-cyclodextrin as additive). For 1:10 diluted samples, k values with different superscript capital letters are significantly different, according to Tukey's HSD test ($p < 0.01$; on the same raw). For 1:10 diluted samples, $t_{1/2}$ values with different superscript lowercase letters are significantly different, according to Tukey's HSD test ($p < 0.05$; on the same raw); Table S2: Kinetic and statistic parameters corresponding to the zeroth–fourth-order models for the "slow" DPPH· reaction with antioxidant compounds from the 1:10 diluted extracts obtained from the fresh and dehydrated strawberries (without or with β-cyclodextrin as additive). For 1:10 diluted samples, k values with different superscript capital letters are significantly different, according to Tukey's HSD test ($p < 0.001$; on the same raw). For 1:10 diluted samples, $t_{1/2}$ values with different superscript lowercase letters are significantly different, according to Tukey's HSD test ($p < 0.01$; on the same raw); Table S3: Kinetic and statistic parameters corresponding to the zeroth–fourth-order models for the "fast" DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh raspberries (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). On the same row, k values with different superscript capital letters are significantly different, according to Tukey's HSD test ($p < 0.10$). On the same row, $t_{1/2}$ values with different superscript lowercase letters are significantly different, according to Tukey's HSD test ($p < 0.15$); Table S4: Kinetic and statistic parameters corresponding to the zeroth–fourth-order models for the "slow" DPPH· reaction with antioxidant compounds from the extracts obtained from the fresh raspberries (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). On the same row, k values with different superscript capital letters are significantly different, according to Tukey's HSD test ($p < 0.05$). On the same row, $t_{1/2}$ values with different superscript lowercase letters are significantly different, according to Tukey's HSD test ($p < 0.05$); Table S5: Kinetic and statistic parameters corresponding to the zeroth–fourth-order models for the "fast" DPPH· reaction with standard 0.4 mM cyanidin 3-*O*-glucoside (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). On the same row, k values with different superscript capital letters are significantly different, according to Tukey's HSD test ($p < 0.05$). On the same row, $t_{1/2}$ values with different superscript lowercase letters are significantly different, according to Tukey's HSD test ($p < 0.05$); Table S6: Kinetic and statistic parameters corresponding to the zeroth–fourth-order models for the "slow" DPPH· reaction with standard 0.4 mM cyanidin 3-*O*-glucoside (without or with β-cyclodextrin as additive during the spectrophotometric monitoring). On the same row, k values with different superscript capital letters are significantly different, according to Tukey's HSD test ($p < 0.05$). On the same row, $t_{1/2}$ values with different superscript lowercase letters are significantly different, according to Tukey's HSD test ($p < 0.15$).

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Abbreviations

The following abbreviations are used in this manuscript:

α-CD	α-Cyclodextrin
Abs	Absorbance (in UV-Vis spectrophotometry)
$ABTS^{+}\cdot$	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
AP	Antioxidant power
ASA	Acetylsalicylic acid
β-CD	β-Cyclodextrin
CD	Cyclodextrin
Cy3G	Cyanidin 3-*O*-glucopyranoside
DPPH·	2,2-Diphenyl-1-picrylhydrazyl radical
dw	dry weight
EC_{50}	Half maximal effective concentration
F	Fisher-value (Fisher-test)
FRAP	Ferric reducing antioxidant power
γ-CD	γ-Cyclodextrin
GRAS	Generally recognized as safe
GSH	Glutathione
HSD	Honestly significant difference (Tukey's HSD test)
k_n	Reaction rate constant for the nth order kinetic model
HPLC	High pressure liquid chromatography
LOD	Limit of detection
LogP	Logarithm of the 1-octanol/water partition coefficient
LOQ	Limit of quantitation
M	Molar concentration, mol/L
mM	milimolar concentration, mmol/L
NaDES	Natural deep eutectic solvent
ORAC	Oxygen radical absorbance capacity
p-value	probability (null-hypothesis significance test)
PEF	Pulsed electric field
Plg3G	Pelargonidin 3-*O*-glucopyranoside
QSAR	Quantitative structure-activity relationship
r/r^2	Correlation/determination coefficient
RB	Raspberry (*Rubus idaeus* L.)
RP-HPLC	Reversed phase—high pressure liquid chromatography
RSA	Radical scavenging activity
RT	Retention time
s	standard error of estimate
SB	Strawberry (*Fragaria* × *ananassa* Duch.)
SD	Standard deviation
$t_{1/2(n)}$	Half-time for the nth order kinetic model
TAC	Total anthocyanin content
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluoroacetic acid
TPC	Total phenolic content
UV-Vis	Ultraviolet-visible spectrophotometry

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References

1. Neha, K.; Haider, M.R.; Pathak, A.; Yar, M.S. Medicinal prospects of antioxidants: A review. *Eur. J. Med. Chem.* **2019**, *178*, 687–704. [CrossRef] [PubMed]
2. Mohammed, D.M.; Maan, S.A.; Baker, D.H.A.; Abozed, S.S. In vitro assessments of antioxidant, antimicrobial, cytotoxicity and anti-inflammatory characteristics of flavonoid fractions from flavedo and albedo orange peel as novel food additives. *Food Biosci.* **2024**, *62*, 105581. [CrossRef]
3. Hussain, A.; Batool, A.; Yaqub, S.; Iqbal, A.; Kauser, S.; Arif, M.R.; Ali, S.; Gorsi, F.I.; Nisar, R.; Hussain, A.; et al. Effects of spray drying and ultrasonic assisted extraction on the phytochemicals, antioxidant and antimicrobial activities of strawberry fruit. *Food Chem. Adv.* **2024**, *5*, 100755. [CrossRef]
4. Silva, F.K.d.S.; Costa-Orlandi, C.B.; Fernandes, M.A.; Brasil, G.S.A.P.; Mussagy, C.U.; Scontri, M.; Sasaki, J.C.d.S.; Abreu, A.P.d.S.; Guerra, N.B.; Floriano, J.F.; et al. Biocompatible anti-aging face mask prepared with curcumin and natural rubber with antioxidant properties. *Int. J. Biol. Macromol.* **2023**, *242*, 124778. [CrossRef]
5. Oyenihi, A.B.; Smith, C. Are polyphenol antioxidants at the root of medicinal plant anti-cancer success? *J. Ethnopharmacol.* **2019**, *229*, 54–72. [CrossRef]
6. Wang, S.; Meckling, K.A.; Marcone, M.F.; Kakuda, Y.; Tsao, R. Can phytochemical antioxidant rich foods act as anti-cancer agents? *Food Res. Int.* **2011**, *44*, 2545–2554. [CrossRef]
7. Mao, X.-Y.; Jin, M.-Z.; Chen, J.-F.; Zhou, H.-H.; Jin, W.-L. Live or let die: Neuroprotective and anti-cancer effects of nutraceutical antioxidants. *Pharmacol. Ther.* **2018**, *183*, 137–151. [CrossRef]
8. Masaki, H. Role of antioxidants in the skin: Anti-aging effects. *J. Dermatol. Sci.* **2010**, *58*, 85–90. [CrossRef]
9. Ghorbani, A. Mechanisms of antidiabetic effects of flavonoid rutin. *Biomed. Pharmacother.* **2017**, *96*, 305–312. [CrossRef]
10. Tolmie, M.; Bester, M.J.; Serem, J.C.; Nell, M.; Apostolides, Z. The potential antidiabetic properties of green and purple tea [*Camellia sinensis* (L.) O Kuntze], purple tea ellagitannins, and urolithins. *J. Ethnopharmacol.* **2023**, *309*, 116377. [CrossRef]
11. Ali, A.; Cottrell, J.J.; Dunshea, F.R. Identification and characterization of anthocyanins and non-anthocyanin phenolics from Australian native fruits and their antioxidant, antidiabetic, and anti-Alzheimer potential. *Food Res. Int.* **2022**, *162*, 111951. [CrossRef]
12. Zhu, M.; Bao, J.; Liang, P.; Zhang, C.; Li, S.; Hu, F. Extraction of flavonoids and phenolic acids from poplar-type propolis: Optimization of maceration process, evaluation of antioxidant and antimicrobial activities. *LWT—Food Sci. Technol.* **2025**, *233*, 118481. [CrossRef]
13. Zhou, P.; Pan, Y.; Yang, W.; Yang, B.; Ou, S.; Liu, P.; Zheng, J. Hepatoprotective effect of cyanidin-3-*O*-glucoside–lauric acid ester against H_2O_2-induced oxidative damage in LO_2 cells. *J. Funct. Foods* **2023**, *107*, 105642. [CrossRef]
14. Bwambale, W.; Oka, V.O.; Etukudo, E.M.; Owu, D.U.; Nkanu, E.E.; Okon, I.A.; Emmanuel, S.D.; Shehu, U.U.; Wilber, N.; Kambere, A.; et al. Nephroprotective activity of naringenin in gentamicin-induced nephrotoxicity in male Wistar rats: In-vivo and in-silico evaluation. *Pharmacol. Res.—Nat. Prod.* **2025**, *9*, 100406. [CrossRef]
15. Martínez-Busi, M.; Arredondo, F.; González, D.; Echeverry, C.; Vega-Teijido, M.A.; Carvalho, D.; Rodríguez-Haralambides, A.; Rivera, F.; Dajas, F.; Abin-Carriquiry, J.A. Purification, structural elucidation, antioxidant capacity and neuroprotective potential of the main polyphenolic compounds contained in *Achyrocline satureioides* (Lam) D.C. (Compositae). *Bioorganic Med. Chem.* **2019**, *27*, 2579–2591. [CrossRef] [PubMed]
16. Kruger, M.J.; Davies, N.; Myburgh, K.H.; Lecour, S. Proanthocyanidins, anthocyanins and cardiovascular diseases. *Food Res. Int.* **2014**, *59*, 41–52. [CrossRef]
17. Mladěnka, P.; Zatloukalová, L.; Filipský, T.; Hrdina, R. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radic. Biol. Med.* **2010**, *49*, 963–975. [CrossRef]
18. Canuto, G.A.B.; Oliveira, D.R.; da-Conceição, L.S.M.; Farah, J.P.S.; Tavares, M.F.M. Development and validation of a liquid chromatography method for anthocyanins in strawberry (*Fragaria* spp.) and complementary studies on stability, kinetics and antioxidant power. *Food Chem.* **2016**, *192*, 566–574. [CrossRef]
19. Chaves, V.C.; Calvete, E.; Reginatto, F.H. Quality properties and antioxidant activity of seven strawberry (*Fragaria* × *ananassa* duch) cultivars. *Sci. Hortic.* **2017**, *225*, 293–298. [CrossRef]
20. Dzhanfezova, T.; Barba-Espín, G.; Müller, R.; Joernsgaard, B.; Hegelund, J.N.; Madsen, B.; Larsen, D.H.; Vega, M.M.; Toldam-Andersen, T.B. Anthocyanin profile, antioxidant activity and total phenolic content of a strawberry (*Fragaria* × *ananassa* Duch) genetic resource collection. *Food Biosci.* **2020**, *36*, 100620. [CrossRef]
21. Nowicka, A.; Kucharska, A.Z.; Sokół-Łętowska, A.; Fecka, I. Comparison of polyphenol content and antioxidant capacity of strawberry fruit from 90 cultivars of *Fragaria* × *ananassa* Duch. *Food Chem.* **2019**, *270*, 32–46. [CrossRef]
22. Hădărugă, D.I.; Hădărugă, N.G. Flavonols. Chemistry, Functionality, and Applications. In *Handbook of Food Bioactive Ingredients: Properties and Applications*; Jafari, S.M., Rashidinejad, A., Simal-Gandara, J., Eds.; Springer Nature Switzerland AG: Cham, Switzerland, 2023; pp. 159–221. [CrossRef]

23. Hădărugă, N.G.; Hădărugă, D.I. Hydroxycinnamic acids. In *Handbook of Food Bioactive Ingredients: Properties and Applications*; Jafari, S.M., Rashidinejad, A., Simal-Gandara, J., Eds.; Springer Nature Switzerland AG: Cham, Switzerland, 2023; pp. 59–109. [CrossRef]
24. Bobinaitė, R.; Viškelis, P.; Venskutonis, P.R. Nutritional Composition of Fruit Cultivars. In *Nutritional Composition of Fruit Cultivars*; Simmonds, M.S.J., Preedy, V.R., Eds.; Elsevier Inc.: Amsterdam, The Netherlands, 2016; pp. 713–731. [CrossRef]
25. de-Souza, V.R.; Pereira, P.A.P.; da-Silva, T.L.T.; Lima, L.C.d.O.; Pio, R.; Queiroz, F. Determination of the bioactive compounds, antioxidant activity and chemical composition of Brazilian blackberry, red raspberry, strawberry, blueberry and sweet cherry fruits. *Food Chem.* **2014**, *156*, 362–368. [CrossRef]
26. Drobek, M.; Cybulska, J.; Zdunek, A.; Sas-Paszt, L.; Frąc, M. Effect of microbial biostimulants on the antioxidant profile, antioxidant capacity and activity of enzymes influencing the quality level of raspberries (*Rubus idaeus* L.). *Food Chem.* **2024**, *454*, 139746. [CrossRef] [PubMed]
27. Sinha, N.K. Strawberries and Raspberries. In *Handbook of Fruits and Fruit Processing*; Sinha, N.K., Sidhu, J.S., Barta, J., Wu, J.S.B., Cano, M.P., Eds.; John Wiley & Sons, Ltd.: Chichester, UK, 2012; pp. 419–431.
28. Wang, Y.; Ye, Y.; Wang, L.; Yin, W.; Liang, J. Antioxidant activity and subcritical water extraction of anthocyanin from raspberry process optimization by response surface methodology. *Food Biosci.* **2021**, *44*, 101394. [CrossRef]
29. de-Souza, V.B.; Fujita, A.; Thomazini, M.; da-Silva, E.R.; Francisco Lucon, J., Jr.; Genovese, M.I.; Favaro-Trindade, C.S. Functional properties and stability of spray-dried pigments from Bordo grape (*Vitis labrusca*) winemaking pomace. *Food Chem.* **2014**, *164*, 380–386. [CrossRef]
30. Krgović, N.; Jovanović, M.S.; Nedeljković, S.K.; Šavikin, K.; Lješković, N.J.; Ilić, M.; Živković, J.; Menković, N. Natural deep eutectic solvents extraction of anthocyanins—Effective method for valorisation of black raspberry (*Rubus occidentalis* L.) pomace. *Ind. Crops Prod.* **2025**, *223*, 120237. [CrossRef]
31. Avalos-Llano, K.R.; Martín-Belloso, O.; Soliva-Fortuny, R. Effect of pulsed light treatments on quality and antioxidant properties of fresh-cut strawberries. *Food Chem.* **2018**, *264*, 393–400. [CrossRef] [PubMed]
32. Lucan-(Banciu), C.A.; Cugerean, M.I.; Gălan, I.M.; Ciobanu-(Şibu), A.; Pop-(Mateuţ), A.; Oprinescu, C.I.; Drăghici, L.R.; Hădărugă, D.I.; Hădărugă, N.G. State-of-the-art on the Dehydration and Drying of *Cucurbita* Species. *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca—Food Sci. Technol.* **2024**, *81*, 1–14. [CrossRef]
33. Asioli, D.; Rocha, C.; Wongprawmas, R.; Popa, M.; Gogus, F.; Almli, V.L. Microwave-dried or air-dried? Consumers' stated preferences and attitudes for organic dried strawberries. A multi-country investigation in Europe. *Food Res. Int.* **2019**, *120*, 763–775. [CrossRef]
34. Amami, E.; Khezami, W.; Mezrigui, S.; Badwaik, L.S.; Bejar, A.K.; Perez, C.T.; Kechaou, N. Effect of ultrasound-assisted osmotic dehydration pretreatment on the convective drying of strawberry. *Ultrason. Sonochemistry* **2017**, *36*, 286–300. [CrossRef]
35. de-Bruijn, J.; Bórquez, R. Quality retention in strawberries dried by emerging dehydration methods. *Food Res. Int.* **2014**, *63*, 42–48. [CrossRef]
36. Contreras, C.; Martín-Esparza, M.E.; Chiralt, A.; Martínez-Navarrete, N. Influence of microwave application on convective drying: Effects on drying kinetics, and optical and mechanical properties of apple and strawberry. *J. Food Eng.* **2008**, *88*, 55–64. [CrossRef]
37. Moreno, J.; Simpson, R.; Pizarro, N.; Parada, K.; Pinilla, N.; Reyes, J.E.; Almonacid, S. Effect of ohmic heating and vacuum impregnation on the quality and microbial stability of osmotically dehydrated strawberries (cv. Camarosa). *J. Food Eng.* **2012**, *110*, 310–316. [CrossRef]
38. Adak, N.; Heybeli, N.; Ertekin, C. Infrared drying of strawberry. *Food Chem.* **2017**, *219*, 109–116. [CrossRef]
39. Avcı, A.S.; Çetinbaş, A.; Karakaya, H. Analysis of strawberry drying behavior under a solar chimney collector using a full factorial method. *Case Stud. Therm. Eng.* **2025**, *73*, 106614. [CrossRef]
40. Doymaz, İ. Convective drying kinetics of strawberry. *Chem. Eng. Process.* **2008**, *47*, 914–919. [CrossRef]
41. El-Beltagy, A.; Gamea, G.R.; Essa, A.H.A. Solar drying characteristics of strawberry. *J. Food Eng.* **2007**, *78*, 456–464. [CrossRef]
42. Jiang, D.-L.; Wang, Q.-H.; Huang, C.; Sutar, P.P.; Lin, Y.-W.; Okaiyeto, S.A.; Lin, Z.-F.; Wu, Y.-T.; Ma, W.-M.; Xiao, H.-W. Effect of various different pretreatment methods on infrared combined hot air impingement drying behavior and physicochemical properties of strawberry slices. *Food Chem. X* **2024**, *22*, 101299. [CrossRef] [PubMed]
43. Zambon, A.; Zulli, R.; Boldrin, F.; Spilimbergo, S. Microbial inactivation and drying of strawberry slices by supercritical CO_2. *J. Supercrit. Fluids* **2022**, *180*, 105430. [CrossRef]
44. Kowalski, S.J.; Pawłowski, A.; Szadzińska, J.; Łechtańska, J.; Stasiak, M. High power airborne ultrasound assist in combined drying of raspberries. *Innov. Food Sci. Emerg. Technol.* **2016**, *34*, 225–233. [CrossRef]
45. Li, S.; Luo, S.; Wang, H.; Liu, H.; Liu, J.; Zhang, X.; Tian, B. Chitosan/polyvinyl alcohol film loading β-acids/β-cyclodextrin inclusion complex: A shelf-life extension strategy for strawberry. *Int. J. Biol. Macromol.* **2025**, *312*, 144223. [CrossRef]
46. Lu, H.; Li, S.; Gao, Q. Development of an antibacterial hydrogel by κ-carrageenan with carvacrol/hydroxypropyl-β-cyclodextrin composite and its application for strawberry preservation. *Food Control* **2025**, *171*, 111100. [CrossRef]

47. Peinado, I.; Rosa, E.; Heredia, A.; Escriche, I.; Andrés, A. Influence of storage on the volatile profile, mechanical, optical properties and antioxidant activity of strawberry spreads made with isomaltulose. *Food Biosci.* **2016**, *14*, 10–20. [CrossRef]
48. Zhang, Y.; Li, F.; Yan, H.; Zhang, Y.; Feng, S.; Deng, L.; Zhao, L.; Gao, S.; Fu, Y.; Ye, F. Nerolidol/hydroxypropyl-*beta*-cyclodextrin inclusion complex nanofibers: Active food packaging for effective strawberry preservation. *Food Chem. X* **2025**, *28*, 102584. [CrossRef] [PubMed]
49. Fan, Y.; Xu, Y.; Wang, D.; Zhang, L.; Sun, J.; Sun, L.; Zhang, B. Effect of alginate coating combined with yeast antagonist on strawberry (*Fragaria* × *ananassa*) preservation quality. *Postharvest Biol. Technol.* **2009**, *53*, 84–90. [CrossRef]
50. Huang, L.-L.; Zhang, M.; Yan, W.-Q.; Mujumdar, A.S.; Sun, D.-F. Effect of coating on post-drying of freeze-dried strawberry pieces. *J. Food Eng.* **2009**, *92*, 107–111. [CrossRef]
51. Wang, H.; Yuan, D.; Meng, Q.; Zhang, Y.; Kou, X.; Ke, Q. Pickering nanoemulsion loaded with eugenol contributed to the improvement of konjac glucomannan film performance. *Int. J. Biol. Macromol.* **2024**, *267*, 131495. [CrossRef]
52. Cerezo, A.B.; Cuevas, E.; Winterhalter, P.; Garcia-Parrilla, M.C.; Troncoso, A.M. Isolation, identification, and antioxidant activity of anthocyanin compounds in *Camarosa* strawberry. *Food Chem.* **2010**, *123*, 574–582. [CrossRef]
53. Agudelo-Laverde, L.M.; Schebor, C.; Buera, M.d.P. Water content effect on the chromatic attributes of dehydrated strawberries during storage, as evaluated by image analysis. *LWT—Food Sci. Technol.* **2013**, *52*, 157–162. [CrossRef]
54. Bórquez, R.M.; Canales, E.R.; Redon, J.P. Osmotic dehydration of raspberries with vacuum pretreatment followed by microwave-vacuum drying. *J. Food Eng.* **2010**, *99*, 121–127. [CrossRef]
55. Angeli, L.; Morozova, K.; Scampicchio, M. A kinetic-based stopped-flow DPPH• method. *Sci. Rep.* **2023**, *13*, 7621. [CrossRef] [PubMed]
56. Kedare, S.B.; Singh, R.P. Genesis and development of DPPH method of antioxidant assay. *J. Food Sci. Technol.* **2011**, *48*, 412–422. [CrossRef]
57. Osugi, J.; Sasaki, M. Kinetic studies on free radical reactions. II. The photochemical reaction between DPPH and methylmethacrylate. *Rev. Phys. Chem. Jpn.* **1964**, *34*, 65–80.
58. Sendra, J.M.; Sentandreu, E.; Navarro, J.L. Reduction kinetics of the free stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) for determination of the antiradical activity of citrus juices. *Eur. Food Res. Technol.* **2006**, *223*, 615–624. [CrossRef]
59. Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative Kinetic Analysis of Hydrogen Transfer Reactions from Dietary Polyphenols to the DPPH Radical. *J. Agric. Food Chem.* **2003**, *51*, 615–622. [CrossRef]
60. Foti, M.C.; Daquino, C.; DiLabio, G.A.; Ingold, K.U. Kinetics of the Oxidation of Quercetin by 2,2-Diphenyl-1-picrylhydrazyl (dpph•). *Org. Lett.* **2011**, *13*, 4826–4829. [CrossRef] [PubMed]
61. Shojaee, M.S.; Moeenfard, M.; Farhoosh, R. Kinetics and stoichiometry of gallic acid and methyl gallate in scavenging DPPH radical as affected by the reaction solvent. *Sci. Rep.* **2022**, *12*, 8765. [CrossRef] [PubMed]
62. Rothwell, J.A.; Day, A.J.; Morgan, M.R.A. Experimental Determination of Octanol-Water Partition Coefficients of Quercetin and Related Flavonoids. *J. Agric. Food Chem.* **2005**, *53*, 4355–4360. [CrossRef]
63. Yanagida, A.; Murao, H.; Ohnishi-Kameyama, M.; Yamakawa, Y.; Shoji, A.; Tagashira, M.; Kanda, T.; Shindo, H.; Shibusawa, Y. Retention behavior of oligomeric proanthocyanidins in hydrophilic interaction chromatography. *J. Chromatogr. A* **2007**, *1143*, 153–161. [CrossRef]
64. Moshari-Nasirkandi, A.; Alirezalu, A.; Hachesu, M.A. Effect of lemon verbena bio-extract on phytochemical and antioxidant capacity of strawberry (*Fragaria* × *ananassa* Duch. cv. Sabrina) fruit during cold storage. *Biocatal. Agric. Biotechnol.* **2020**, *25*, 101613. [CrossRef]
65. Mustafa, A.M.; Angeloni, S.; Abouelenein, D.; Acquaticci, L.; Xiao, J.; Sagratini, G.; Maggi, F.; Vittori, S.; Caprioli, G. A new HPLC-MS/MS method for the simultaneous determination of 36 polyphenols in blueberry, strawberry and their commercial products and determination of antioxidant activity. *Food Chem.* **2022**, *367*, 130743. [CrossRef]
66. Corrales, M.; Lindauer, R.; Butz, P.; Tauscher, B. Effect of heat/pressure on cyanidin-3-glucoside ethanol model solutions. *J. Phys. Conf. Ser.* **2008**, *121*, 142003. [CrossRef]
67. Zhang, Y.; Sun, J.; Hu, X.; Liao, X. Spectral Alteration and Degradation of Cyanidin-3-glucoside Exposed to Pulsed Electric Field. *J. Agric. Food Chem.* **2010**, *58*, 3524–3531. [CrossRef]
68. Ichiyanagi, T.; Oikawa, K.; Tateyama, C.; Konishi, T. Acid Mediated Hydrolysis of Blueberry Anthocyanins. *Chem. Pharm. Bull.* **2001**, *49*, 114–117. [CrossRef]
69. Sadilova, E.; Carle, R.; Stintzing, F.C. Thermal degradation of anthocyanins and its impact on color and in vitro antioxidant capacity. *Mol. Nutr. Food Res.* **2007**, *51*, 1461–1471. [CrossRef]
70. Hădărugă, D.I.; Hădărugă, N.G. Flavanones in Plants and Humans. Chemistry, Functionality, and Applications. In *Handbook of Food Bioactive Ingredients: Properties and Applications*; Jafari, S.M., Rashidinejad, A., Simal-Gandara, J., Eds.; Springer Nature Switzerland AG: Cham, Switzerland, 2023; pp. 223–275. [CrossRef]

71. Hădărugă, N.G.; Hădărugă, D.I. Stilbenes and Its Derivatives and Glycosides. In *Handbook of Food Bioactive Ingredients: Properties and Applications*; Jafari, S.M., Rashidinejad, A., Simal-Gandara, J., Eds.; Springer Nature Switzerland AG: Cham, Switzerland, 2023; pp. 487–544. [CrossRef]
72. Hădărugă, D.I.; Hădărugă, N.G.; Bandur, G.N.; Isengard, H.-D. Water content of flavonoid/cyclodextrin nanoparticles: Relationship with the structural descriptors of biologically active compounds. *Food Chem.* **2012**, *132*, 1651–1659. [CrossRef]
73. Hădărugă, N.G.; Bandur, G.N.; David, I.; Hădărugă, D.I. A review on thermal analyses of cyclodextrins and cyclodextrin complexes. *Environ. Chem. Lett.* **2019**, *17*, 349–373. [CrossRef]
74. Mandave, P.C.; Pawar, P.K.; Ranjekar, P.K.; Mantri, N.; Kuvalekar, A.A. Comprehensive evaluation of in vitro antioxidant activity, total phenols and chemical profiles of two commercially important strawberry varieties. *Sci. Hortic.* **2014**, *172*, 124–134. [CrossRef]
75. Rahman, M.H.; Hasan, M.N.; Khan, M.Z.H. Study on different nano fertilizers influencing the growth, proximate composition and antioxidant properties of strawberry fruits. *J. Agric. Food Res.* **2021**, *6*, 100246. [CrossRef]
76. Wang, J.; Wang, J.; Ye, J.; Vanga, S.K.; Raghavan, V. Influence of high-intensity ultrasound on bioactive compounds of strawberry juice: Profiles of ascorbic acid, phenolics, antioxidant activity and microstructure. *Food Control* **2019**, *96*, 128–136. [CrossRef]
77. Huang, X.; Wu, Y.; Zhang, S.; Yang, H.; Wu, W.; Lyu, L.; Li, W. Changes in antioxidant substances and antioxidant enzyme activities in raspberry fruits at different developmental stages. *Sci. Hortic.* **2023**, *321*, 112314. [CrossRef]
78. Zhang, L.; Li, J.; Hogan, S.; Chung, H.; Welbaum, G.E.; Zhou, K. Inhibitory effect of raspberries on starch digestive enzyme and their antioxidant properties and phenolic composition. *Food Chem.* **2010**, *119*, 592–599. [CrossRef]
79. Falah, M.A.F.; Machfoedz, M.M.; Rahmatika, A.M.; Putri, R.M. Quality characterization of freeze-dried tropical strawberries pretreated through osmotic dehydration. *J. Agric. Food Res.* **2025**, *21*, 101901. [CrossRef]
80. Santos, N.C.; Almeida, R.L.J.; Monteiro, S.S.; Silva, E.T.d.V.; Silva, V.M.d.A.; André, A.M.M.C.N.; Ribeiro, V.H.d.A.; de-Brito, A.C.O. Influence of ethanol and ultrasound on drying, bioactive compounds, and antioxidant activity of strawberries (*Fragaria* × *ananassa*). *J. Indian Chem. Soc.* **2022**, *99*, 100542. [CrossRef]
81. Chat, O.A.; Najar, M.H.; Dar, A.A. Evaluation of reduction kinetics of 2,2-diphenyl-1-picrylhydrazylradical by flavonoid glycoside Rutin in mixed solvent based micellar media. *Colloids Surf. A Physicochem. Eng. Asp.* **2013**, *436*, 343–353. [CrossRef]
82. Foti, M.C.; Slavova-Kazakov, A.; Rocco, C.; Kancheva, V.D. Kinetics of curcumin oxidation by 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$): An interesting case of separated coupled proton–electron transfer. *Org. Biomol. Chem.* **2016**, *14*, 8331–8337. [CrossRef] [PubMed]
83. Iordănescu, O.A.; Băla, M.; Gligor-(Pane), D.; Zippenfening, S.E.; Cugerean, M.I.; Petroman, M.I.; Hădărugă, D.I.; Hădărugă, N.G.; Riviş, M. A DPPH· Kinetic Approach on the Antioxidant Activity of Various Parts and Ripening Levels of Papaya (*Carica papaya* L.) Ethanolic Extracts. *Plants* **2021**, *10*, 1679. [CrossRef]
84. Iordănescu, O.A.; Băla, M.; Iuga, A.C.; Gligor-(Pane), D.; Dascălu, I.; Bujancă, G.S.; David, I.; Hădărugă, N.G.; Hădărugă, D.I. Antioxidant activity and discrimination of organic apples (*Malus domestica* Borkh.) cultivated in the western region of Romania: A DPPH· kinetics–PCA approach. *Plants* **2021**, *10*, 1957. [CrossRef]
85. Jabbari, M.; Jabbari, A. Antioxidant potential and DPPH radical scavenging kinetics of water-insoluble flavonoid naringenin in aqueous solution of micelles. *Colloids Surf. A Physicochem. Eng. Asp.* **2016**, *489*, 392–399. [CrossRef]
86. Pannala, A.S.; Chan, T.S.; O'Brien, P.J.; Rice-Evans, C.A. Flavonoid B-Ring Chemistry and Antioxidant Activity: Fast Reaction Kinetics. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 1161–1168. [CrossRef]
87. Campos, A.M.; Lissi, E.A. Kinetics of the Reaction between 2,2′-Azinobis(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) Derived Radical Cations and Phenols. *Int. J. Chem. Kinet.* **1997**, *29*, 219–224. [CrossRef]
88. Henriquez, C.; Aliaga, C.; Lissi, E. Kinetics profiles in the reaction of ABTS derived radicals with simple phenols and polyphenols. *J. Chil. Chem. Soc.* **2004**, *49*, 65–67. [CrossRef]
89. Pérez-Jiménez, J.; Saura-Calixto, F. Anti-oxidant capacity of dietary polyphenols determined by ABTS assay: A kinetic expression of the results. *Int. J. Food Sci. Technol.* **2008**, *43*, 185–191. [CrossRef]
90. Hădărugă, N.-G.; Popescu, G.; Gligor-(Pane), D.; Mitroi, C.L.; Stanciu, S.M.; Hădărugă, D.I. Discrimination of β-cyclodextrin/hazelnut (*Corylus avellana* L.) oil/flavonoid glycoside and flavonolignan ternary complexes by Fourier-transform infrared spectroscopy coupled with principal component analysis. *Beilstein J. Org. Chem.* **2023**, *19*, 380–398. [CrossRef] [PubMed]
91. Horablaga, A.; Şibu-(Ciobanu), A.; Megyesi, C.I.; Gligor-(Pane), D.; Bujancă, G.S.; Velciov, A.B.; Morariu, F.E.; Hădărugă, D.I.; Mişcă, C.D.; Hădărugă, N.G. Estimation of the Controlled Release of Antioxidants from β-Cyclodextrin/Chamomile (*Matricaria chamomilla* L.) or Milk Thistle (*Silybum marianum* L.), Asteraceae, Hydrophilic Extract Complexes through the Fast and Cheap Spectrophotometric Technique. *Plants* **2023**, *12*, 2352. [CrossRef]
92. Cai, R.; Yuan, Y.; Cui, L.; Wang, Z.; Yue, T. Cyclodextrin-assisted extraction of phenolic compounds: Current research and future prospects. *Trends Food Sci. Technol.* **2018**, *79*, 19–27. [CrossRef]
93. You, G.-J.; Sun, L.-L.; Cao, X.-X.; Li, H.-H.; Wang, M.; Liu, Y.-N.; Ren, X.-L. Comprehensive evaluation of solubilization of flavonoids by various cyclodextrins using high performance liquid chromatography and chemometry. *LWT—Food Sci. Technol.* **2018**, *94*, 172–177. [CrossRef]

94. Lucas-Abellán, C.; Pérez-Abril, M.; Castillo, J.; Serrano, A.; Mercader, M.T.; Fortea, M.I.; Gabaldón, J.A.; Núñez-Delicado, E. Effect of temperature, pH, β- and HP-β-cds on the solubility and stability of flavanones: Naringenin and hesperetin. *LWT—Food Sci. Technol.* **2019**, *108*, 233–239. [CrossRef]
95. Ratnasooriya, C.C.; Rupasinghe, H.P.V. Extraction of phenolic compounds from grapes and their pomace using β-cyclodextrin. *Food Chem.* **2012**, *134*, 625–631. [CrossRef] [PubMed]
96. Krstić, L.; Jarho, P.; Ruponen, M.; Urtti, A.; González-García, M.J.; Diebold, Y. Improved ocular delivery of quercetin and resveratrol: A comparative study between binary and ternary cyclodextrin complexes. *Int. J. Pharm.* **2022**, *624*, 122028. [CrossRef]
97. Nguyen, T.A.; Liu, B.; Zhao, J.; Thomas, D.S.; Hook, J.M. An investigation into the supramolecular structure, solubility, stability and antioxidant activity of rutin/cyclodextrin inclusion complex. *Food Chem.* **2013**, *136*, 186–192. [CrossRef]
98. Pinhoa, E.; Grootveld, M.; Soares, G.; Henriques, M. Cyclodextrins as encapsulation agents for plant bioactive compounds. *Carbohydr. Polym.* **2014**, *101*, 121–135. [CrossRef]
99. Xiong, Y.; Chang, M.; Shi, Z.-W.; Li, Y.-Y.; An, S.-Y.; Ren, D.-F. Encapsulation of rose anthocyanins with β-cyclodextrin for enhanced stability: Preparation, characterization, and its application in rose juice. *Food Biosci.* **2025**, *64*, 105875. [CrossRef]
100. Mourtzinos, I.; Makris, D.P.; Yannakopoulou, K.; Kalogeropoulos, N.; Michali, I.; Karathanos, V.T. Thermal Stability of Anthocyanin Extract of *Hibiscus sabdariffa* L. in the Presence of β-Cyclodextrin. *J. Agric. Food Chem.* **2008**, *56*, 10303–10310. [CrossRef]
101. Xie, J.; Xu, Y.; Shishir, M.R.I.; Zheng, X.; Chen, W. Green extraction of mulberry anthocyanin with improved stability using β-cyclodextrin. *J. Sci. Food Agric.* **2019**, *99*, 2494–2503. [CrossRef]
102. Peron, D.V.; Fraga, S.; Antelo, F. Thermal degradation kinetics of anthocyanins extracted from juçara (*Euterpe edulis* Martius) and "Italia" grapes (*Vitis vinifera* L.), and the effect of heating on the antioxidant capacity. *Food Chem.* **2017**, *232*, 836–840. [CrossRef]
103. Kim, A.-N.; Kim, H.-J.; Chun, J.; Heo, H.J.; Kerr, W.L.; Choi, S.-G. Degradation kinetics of phenolic content and antioxidant activity of hardy kiwifruit (*Actinidia arguta*) puree at different storage temperatures. *LWT—Food Sci. Technol.* **2018**, *89*, 535–541. [CrossRef]
104. Rodríguez-Ramírez, J.; Méndez-Lagunas, L.L.; López-Ortiz, A.; Muñiz-Becerá, S.; Nair, K. Solar drying of strawberry using polycarbonate with UV protection and polyethylene covers: Influence on anthocyanin and total phenolic content. *Sol. Energy* **2021**, *221*, 120–130. [CrossRef]
105. Méndez-Lagunas, L.; Rodríguez-Ramírez, J.; Cruz-Gracida, M.; Sandoval-Torres, S.; Barriada-Bernal, G. Convective drying kinetics of strawberry (*Fragaria ananassa*): Effects on antioxidant activity, anthocyanins and total phenolic content. *Food Chem.* **2017**, *230*, 174–181. [CrossRef] [PubMed]
106. Méndez-Lagunas, L.L.; Rodríguez-Ramírez, J.; Sandoval-Torres, S.; Barragán-Iglesias, J.; López-Ortíz, A. Strawberry fluidized bed drying. Antiadhesion pretreatments and their effect on bioactive compounds. *Appl. Food Res.* **2025**, *5*, 100739. [CrossRef]
107. Stojiljković, D.; Arsić, I.; Tadić, V. Extracts of wild apple fruit (*Malus sylvestris* (L.) Mill., Rosaceae) as asource of antioxidant substances for use in production ofnutraceuticals and cosmeceuticals. *Ind. Crops Prod.* **2016**, *80*, 165–176. [CrossRef]
108. Veggi, P.C.; Santos, D.T.; Meireles, M.A.A. Anthocyanin extraction from Jabuticaba (*Myrciaria cauliflora*) skins by different techniques: Economic evaluation. *Procedia Food Sci.* **2011**, *1*, 1725–1731. [CrossRef]
109. Zhang, Z.; Kou, X.; Fugal, K.; McLaughlin, J. Comparison of HPLC Methods for Determination of Anthocyanins and Anthocyanidins in Bilberry Extracts. *J. Agric. Food Chem.* **2004**, *52*, 688–691. [CrossRef] [PubMed]
110. Ghose, A.K.; Pritchett, A.; Crippen, G.M. Atomic Physicochemical Parameters for Three Dimensional Structure Directed Quantitative Structure–Activity Relationships III: Modeling Hydrophobic Interactions. *J. Comput. Chem.* **1988**, *9*, 80–90. [CrossRef]
111. Viswanadhan, V.N.; Ghose, A.K.; Reyankar, G.R.; Robins, R.K. Atomic Physicochemical Parameters for Three Dimensional Structure Directed Quantitative Structure–Activity Relationships. 4. Additional Parameters for Hydrophobic and Dispersive Interactions and Their Application for an Automated Superposition of Certain Naturally Occurring Nucleoside Antibiotics. *J. Chem. Inf. Comput. Sci.* **1989**, *29*, 163–172. [CrossRef]

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