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Evaluation of antibacterial, antioxidant activities and secondary metabolites profile of orchids with different dried methods

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ABSTRACT: The Central Black Sea region is a very dense region in terms of orchid diversity. As a consequence of the floral studies, 32 species of salep orchids were identified in the Central Black Sea Region. The aim of this current study was to identify the secondary metabolite profiles of leaves and tubers of two orchid species (*Anacamptis palustris* and *Ophrys apifera*) with different drying methods by HPLC analysis and to determine their antibacterial and antioxidant activities. For this purpose, different drying methods were applied to the roots and leaves of these two species. The species were dried by 3 different techniques: shade, lyophilizer and oven. After the aqueous extracts were obtained, their antibacterial and antioxidant activities were tested and HPLC analysis was completed. The data obtained from aqueous extracts as a result of drying techniques were compared with the data obtained from fresh plant aqueous extracts. Antibacterial properties were tested against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 bacteria. No antibacterial activity was found in the study. Antioxidant properties were tested via DPPH and FRAP assays. As a result of DPPH free radical scavenging assay, the highest antioxidant efficiency in *A. palustris* was observed in leaves dried in lyophilizer (76.53%), in the shade (62.13%) and in the oven (54.12%), respectively. Nine different phenolic compounds were found in aqueous extracts of these species. Epicatechin, Gallic Acid, Rutin, Syringic Acid phenolic compounds were observed the most in HPLC analysis. The results of current study suggest that these extracts have antioxidant and phenolic contents and should be investigated in more comprehensive studies.

KEYWORDS: *Anacamptis palustris*; *Ophrys apifera*; Drying techniques; HPLC analysis; Antioxidant activity.

1. INTRODUCTION

Orchids are one of the biggest families of flowering plants and belong to the Orchidaceae family [1, 2]. The Orchidaceae family, which is spread over a wide geography in the world, has approximately 25,000 species [3-5]. The most significant genera include Orchis, Himantoglossum, Anacamptis, Serapias, Barlia, Compreria, Aceras, Dactylorhiza, Neotinea, and Ophrys. Approximately 120 orchid taxa are currently utilized in the manufacture of salep in Türkiye [6]. The Central Black Sea region is a very dense region in terms of orchid diversity. As a consequence of the floral studies, 32 species of salep orchids were identified in the Central Black Sea Region [7]. All components of orchids, including leaves, flowers, roots, pseudobulbs, rhizomes, tubers, and entire plants, are used in medicine. Polysaccharides, alkaloids, glycosides, phenolic compounds, and many other secondary metabolites are among the medicinally active substances found in orchid tissues [5]. In Türkiye, Iran and Greece, the Eurasian orchids tubers are harvested as food addeditives due to their glucomannan ingredient. In eastern Türkiye, salep is used to cure diarrhea and bronchitis [8]. Orchid species are going extinct as a result of their abuse in the production of salep.

Anacamptis palustris is a marsh orchid in the Orchidaceae family. Only marsh conditions are suitable for the species' growth, usually in retrodunal regions along the coast and infrequently in extremely salinized locations like sulfuric springs. Because the present spread of *A. palustris* is highly patchy, the species is listed

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as seriously endangered [9]. Another orchid species, *Ophrys apifera*, is a rare orchid with flowers that mimic the shape of a bee to attract pollinators. Flowering time is May-June. Like most orchids, it is small and does not spread easily [10]. Ethnobotanically, nutritional and medicinal uses of *O. apifera* tubers have been reported in the literature, mostly related to salep production [11]. In some regions, medicinal uses of tubers have been recorded for the treatment of inflammatory gastrointestinal and urogenital diseases [12].*O. apifera* was also reported to have a high concentration of flavonoid compounds [13].

Drying is a critical step in the post-harvest processing of herbs, medicinal and spices plants and has a decisive role in determining the quality of the dried product. The advantages of drying include keeping the moisture content at a level that prevents microbial and chemical activities during storage, preventing deterioration in terms of active ingredients, flavor, color and aroma, and preventing microbial growth [14]. Drying processes have been reported to have significant effects on the content of bioactive components and antioxidant properties [15]. Microwave, infrared, sun, hot air, vacuum, spray, etc. are among the drying techniques [16].

In the literatures, various solvents such as hexane, dichloromethane, ethyl acetate and methanol were used to obtain herbal extracts [17]. Water is the greenest and renewable solvent. After proper treatment, aqueous wastes can be released because they are non-flammable and non-toxic. In order to obtain aqueous extracts, any methodology that used water or aqueous-based media was chosen because it makes a process more environmentally friendly [18]. Many solvent combinations are employed to extract antioxidant compounds from plant materials, including fruits, vegetables, and other meals. Ethanol, methanol, water, acetone, and their acidic or non-acidic water mixes are the most often utilized solvents for the phenolic compounds extraction. The solvent utilized has a significant impact on the extracts' antioxidant potential. The antioxidant qualities of fruits and vegetables are typically attributed to phenolic chemicals, the majority of which are categorized as hydrophilic antioxidants [19]. Ethanol was generally used in extraction studies of the Orchidaceae family [20, 21]. However, ethnobotanical studies have indicated that water extracts are traditionally used by the people [22-26]. In biological activity studies of methanol and water extractions, it was stated that methanol extracts showed the highest activity compared to aqueous extracts [27]. In addition, it was reported that some aqueous extracts showed antibacterial activity while others did not show any activity [28].

The World Health Organization (WHO) reported that 25% of pharmaceutical drugs are made from medicinal plants. Natural products are endless sources of compounds used in the fight against various pathologies [29]. High amounts of phenolic compounds with potent antioxidant and antibacterial properties can be found in natural components like plant extracts [30]. The use of plant-derived extracts as possible medicines to fight microbial infections has gained more attention recently. The primary reason given was the growing demand from consumers for environmentally friendly products. Reports indicate that using extracts from plants can efficiently eliminate bacteria and enzymes while having little negative impact on the treated substance's sensory or nutritional qualities [31]. The aim of this current study was to identify the secondary metabolite profiles of leaves and tubers of two orchid species (*Anacamptis palustris* and *Ophrys apifera*) with different drying methods (shade, lyophilizer and oven) by HPLC analysis and to determine their antibacterial and antioxidant activities.

2. RESULTS AND DISCUSSION

In our study, aqueous extracts of leaves and tubers of *A. palustris* and *O. apifera* species dried by different methods were obtained, secondary metabolite profiles were revealed by HPLC analysis, and antioxidant and antibacterial effects were tested.

2.1. HPLC Analysis

In HPLC analysis, 22 different phenolic compounds were investigated, including 4-Hydroxybenzoic Acid, Caffeic Acid, Crysin, Quercetin, Gallic Acid, Protocatechuic Acid, Sesamol, P. Coumaric Acid, Epicatechin, Rosmarinic Acid, Rutin, Benzoic Acid, t-cinnamic Acid, Kaempherol, Catechin, Vanillic Acid, Vanilin, Syringaldehyde, Protocatechuic aldehyde, Ferulic Acid, Syringic Acid, Coumarin. As a result of the analysis, nine different phenolic compounds were encountered in aqueous extracts of *A. palustris* and *O. apifera*. The amounts and types of these phenolic compounds differed according to the drying technique and plant part. As a result of HPLC analysis, Catechin, Epicatechin, Gallic Acid, P. Coumaric Acid, Protocatechuic Aldehyde, Rutin, Syringic Acid, t-cinnamic Acid, Vanillin phenolic compounds were observed, respectively.

In our study on *A. palustris* aqueous leaf extract, Gallic acid (78.00 µg/g), P. Coumaric Acid (59.20 µg/g), Protocatechuic Aldehyde (2.00 µg/g) and Syringic Acid (30.40 µg/g) were detected in fresh leaf. However, Gallic acid (261.60 µg/g), P. Coumaric Acid (209.20 µg/g), Protocatechuic Aldehyde (76.80 µg/g), Rutin (1812.40 µg/g), Syringic Acid (792.00 µg/g), t-cinnamic Acid (62.40 µg/g) were detected after drying in the oven. In shade drying, Gallic acid (394.00 µg/g), P. Coumaric Acid (223.20 µg/g), Protocatechuic Aldehyde (69.60 µg/g), Rutin (3006.00 µg/g), Syringic Acid (808.00 µg/g) were detected. As a result of lyophilized drying, Epicatechin (222.40 µg/g), Gallic acid (510.80 µg/g), P. Coumaric Acid (168.40 µg/g), Protocatechuic Aldehyde (52.80 µg/g), Rutin (1858.80 µg/g), Syringic Acid (431.20 µg/g) were found. On aqueous tuber extract, Gallic acid (50.40 µg/g), P. Coumaric Acid (43.60 µg/g), Rutin (112.40 µg/g), Syringic Acid (164.80 µg/g) were detected in fresh tuber. However, after drying in the oven, Catechin (418.00 µg/g), Epicatechin (169.60 µg/g), Gallic acid (207.20 µg/g), P. Coumaric Acid (74.00 µg/g), Rutin (226.80 µg/g), Syringic Acid (1802.00 µg/g), t-cinnamic Acid (45.60 µg/g) and Vanillin (10.40 µg/g) were determined. In shade drying, Epicatechin (128.80 µg/g), Gallic acid (286.00 µg/g), P. Coumaric Acid (135.60 µg/g), Rutin (429.20 µg/g), Syringic Acid (1599.60 µg/g), t-cinnamic Acid (43.60 µg/g) and Vanillin (3.20 µg/g) were detected. As a result of lyophilized drying, Epicatechin (123.20 µg/g), Gallic acid (151.60 µg/g), P. Coumaric Acid (84.00 µg/g), Rutin (1279.60 µg/g), Syringic Acid (1705.20 µg/g), t-cinnamic Acid (142.80 µg/g) and Vanillin (40.40 µg/g) were found. Figure 1 showed the phenolic compounds observed as a result of HPLC analysis depending on different drying techniques of *A. palustris*. Rutin, one of these phenolic compounds, was most abundant in the shade, then in the lyophilizer and finally in the oven-dried leaves, respectively. Syringic acid was observed most intensively in tubers dried in the oven, then in the lyophilizer and finally in the shade, respectively.

In our study on O. apifera aqueous leaf extract, Gallic acid (69.75 µg/g), P. Coumaric Acid (51.42 µg/g), Protocatechuic Aldehyde (79.60 µg/g) and Rutin (41.62 µg/g) were detected in fresh leaf. However, after drying in the oven, Gallic acid (475.37 µg/g), P. Coumaric Acid (68.18 µg/g), Protocatechuic Aldehyde (467.55 µg/g), Rutin (1152.86 µg/g), Syringic Acid (1076.22 µg/g), t-cinnamic Acid (294.17 µg/g) were determined. In shade drying, Gallic acid (436.39 µg/g), P. Coumaric Acid (157.54 µg/g), Protocatechuic Aldehyde (389.75 µg/g), Rutin (1294.10 µg/g), Syringic Acid (1016.27 µg/g), t-cinnamic Acid (143.40 µg/g) were determined. As a result of lyophilized drying, Epicatechin (731.17 µg/g), Gallic acid (626.45 µg/g), P. Coumaric Acid (150.32 µg/g), Protocatechuic Aldehyde (251.38 µg/g), Rutin (1320.67 µg/g), Syringic Acid (1461.67 µg/g), t-cinnamic Acid (141.92 µg/g) were found. On aqueous tuber extract, Gallic acid (47.20 µg/g), P. Coumaric Acid (36.21 µg/g), Syringic Acid (42.46 µg/g) and t-cinnamic Acid (33.00 µg/g) were determined in fresh tuber. However, as a result of drying in the oven, Catechin (412.02 µg/g), Epicatechin (176.75 µg/g), Gallic acid (237.53 µg/g), P. Coumaric Acid (54.30 µg/g), Protocatechuic Aldehyde (10.01 µg/g), Rutin (251.49 µg/g), Syringic Acid (1169.34 µg/g), t-cinnamic Acid (51.53 µg/g) and Vanillin (42.19 µg/g) were detected. In shade drying, Epicatechin (731.17 µg/g), Gallic acid (626.45 µg/g), P. Coumaric Acid (150.32 µg/g), Protocatechuic Aldehyde (251.38 µg/g), Rutin (1320.67 µg/g), Syringic Acid (1461.67 µg/g), t-cinnamic Acid (141.92 µg/g) were detected. As a result of lyophilized drying, Catechin (226.86 µg/g), Epicatechin (193.81 µg/g), Gallic acid (225.90 µg/g), P. Coumaric Acid (79.64 µg/g), Syringic Acid (603.47 µg/g) were found. Figure 2 showed the phenolic compounds observed as a result of HPLC analysis of *O. apifera* species depending on different drying techniques. Syringic acid, one of these phenolic compounds, was observed most intensely in the tuber dried in the shade, then in the leaf dried in the lyophilizer and finally in the tuber dried in the oven. Rutin was most abundant in the leaf dried in the lyophilizer, then in the shade and finally in the oven-dried leaf, respectively. Epicatechin was most abundant in the leaf dried in the lyophilizer.

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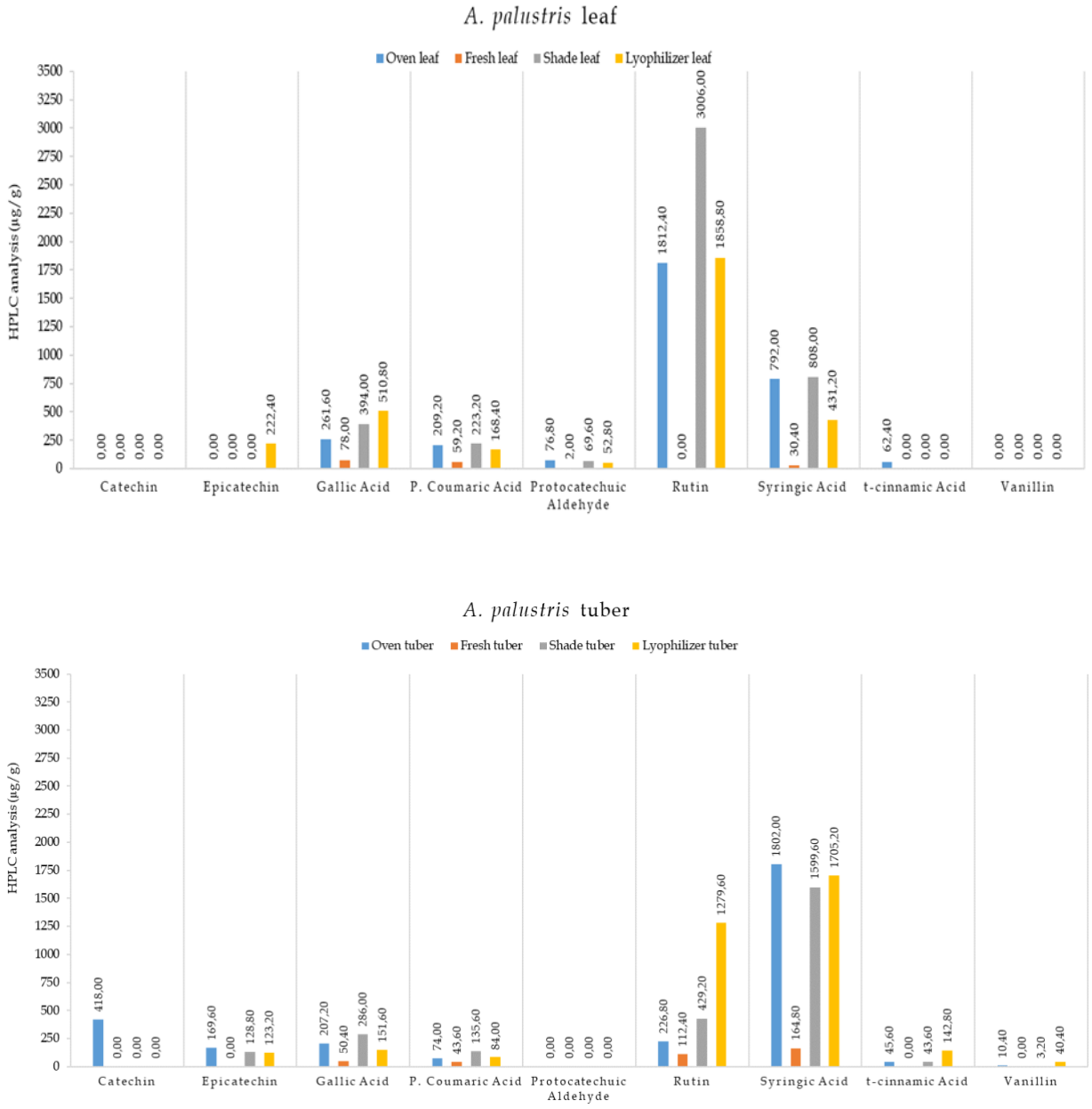

Figure 1. HPLC anaylsis of *A. palustris* extract

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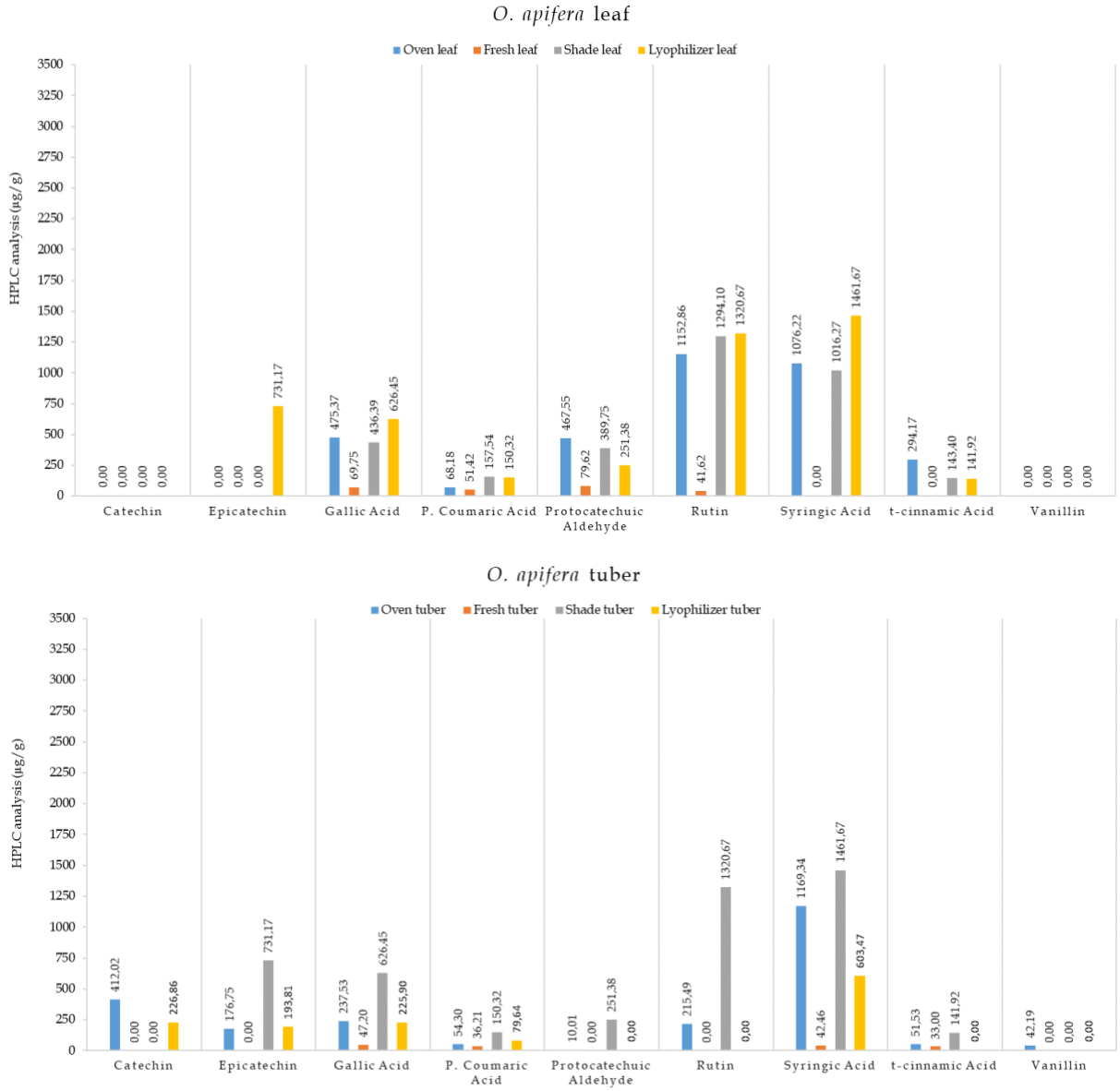

Figure 2. HPLC anaylsis of *O. apifera* extract

It is known that plant secondary metabolites have different bioactive properties, especially antioxidant effects [32]. HPLC analysis shows that especially extracts obtained from fresh leaves and fresh tubers have lower polyphenolic content than other extracts, and when the results of antioxidant analyses are examined, it is seen that they have lower antioxidant activity than other extracts. It has been observed that polyphenolic compounds isolated from species by extraction have a positive effect on antioxidant capacities. As a result, it was observed that *A. palustris* plant had more intense amounts of phenolic compounds compared to *O. apifera* plant. However, the amounts of Epicatechin and Gallic Acid were found in higher amounts in *O. apifera* plant.

Medicinal flora have constituted a fundamental component of conventional therapeutic practices since antiquity. Flavonoids, which are polyphenolic compounds, represent a significant category of phytochemicals that encompass the benzopyrone moiety. Approximately 4000 distinct varieties of flavonoids have been documented as existing within various plant species. In the present investigation, syringin acid and rutin were identified as the predominant bioactive constituents within the plant specimens, as evidenced by the data derived from high-performance liquid chromatography (HPLC) analysis. Syringic acid is categorized as a phenolic compound typically present in various vegetables and fruits, and it is biosynthesized in plants through the metabolic pathway involving syringic acid. This

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particular compound exhibits a broad spectrum of therapeutic potential in the prophylaxis of diabetes, oncological conditions, cardiovascular ailments, and cerebral ischemia; furthermore, it demonstrates significant antioxidant, antimicrobial, anti-inflammatory, antiendotoxic, as well as neuroprotective and hepatoprotective properties [33]. Rutin, an additional bioactive compound discerned during the analytical process, is acknowledged in over 70 distinct plant species and is present in various plant-derived food products. The pharmacological attributes of rutin encompass a wide array of advantageous activities, including antifungal, anticancer, antibacterial, anti-inflammatory, and antioxidant effects, in addition to neuroprotective and cardioprotective benefits [34]. Consequently, it is posited that the observed activities are attributable to these primary constituents. Conversely, secondary constituents are also plausibly implicated in these activities.

2.2. Antioxidant Activity

The antioxidant activities of *A. palustris* and *O. apifera* species leaves and tubers with different drying techniques were tested by DPPH and FRAP assays.

As a result of FRAP assay, the highest antioxidant activity in *A. palustris* was observed in leaves dried in the lyophilizer (44.40 mgTE/g sample), in the shade (41.10 mgTE/g sample) and in the oven (32.83 mgTE/g sample), respectively. However, the lowest antioxidant activity was observed in fresh leaves (5.69 mgTE/g sample). In tubers, the highest antioxidant activity was observed in tubers dried in the shade (2.53 mgTE/g sample), in the lyophilizer (1.86 mgTE/g sample) and in the oven (1.79 mgTE/g sample), respectively. However, the lowest antioxidant activity was observed in fresh tubers (0.33 mgTE/g sample). As a result of DPPH free radical scavenging assay, the highest antioxidant efficiency in *A. palustris* was observed in leaves dried in lyophilizer (76.53%), in the shade (62.13%) and in the oven (54.12%), respectively. However, the lowest antioxidant activity was observed in fresh leaves (11.73%). In tubers, the highest antioxidant efficiency was observed in tubers dried in shade (26.45%), in the oven (17.64%) and in the lyophilizer (17.63%), respectively. However, the lowest antioxidant efficiency was observed in fresh tuber (6.87%). Results shown in Figure 3 and Table 1.

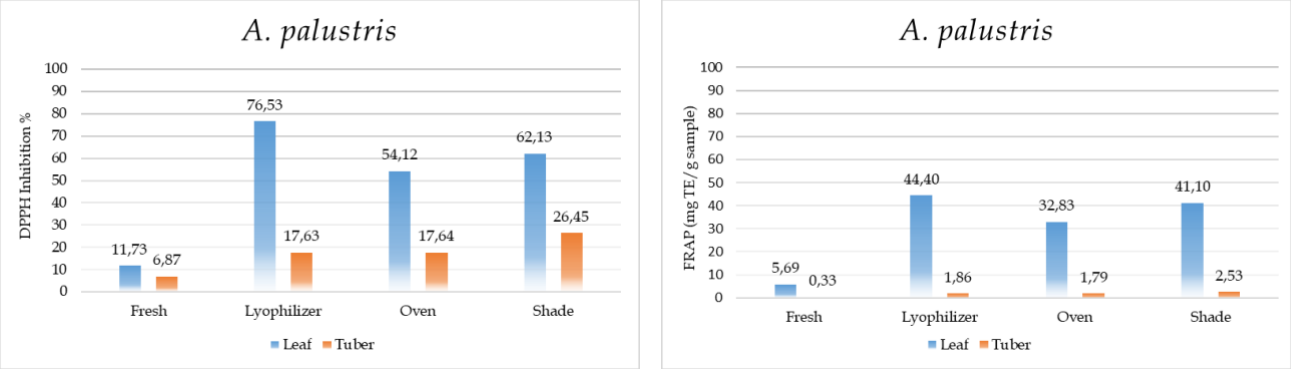

Figure 3. Effect of drying techniques on *O. apifera* and *A. palustris* antioxidant activity

As a result of FRAP assay, the highest antioxidant activity in *O. apifera* was observed in leaves dried in the lyophilizer (38.60 mgTE/g sample), in the shade (35.59 mgTE/g sample) and in the oven (34.01 mgTE/g sample), respectively. However, the lowest antioxidant activity was observed in fresh leaves (3.52 mgTE/g sample). In tubers, the highest antioxidant activity was observed in tubers dried in the oven (6.73 mgTE/g sample), in the shade (4.26 mgTE/g sample) and in the lyophilizer (2.31 mgTE/g sample), respectively. However, the lowest antioxidant activity was observed in fresh tubers (0.13 mgTE/g sample). As a result of DPPH free radical scavenging assay, the highest antioxidant activity in *O. apifera* was observed in leaves dried in lyophilizer (54.36%), shade (46.19%) and oven (35.19%), respectively. However, the lowest antioxidant activity was observed in fresh leaves (6.87%). In tubers, the highest antioxidant activity was observed in tubers dried in oven (41.50%), shade (34.14%) and lyophilizer (16.83%), respectively. However, the lowest antioxidant activity was observed in fresh tuber (4.53%). Results shown in Figure 4 and Table 1.

Statistical data of the obtained antioxidant activities results are shown in Table 1.

Table 1. Statistical data on antioxidant activity results obtained depending on drying techniques

		FRAP (mg TE/gram sample)		DPPH Inhibition %	
	Drying technique	Leaf	Tuber	Leaf	Tuber
A. palustris	Fresh	5.68 ± 0.105^{g}	0.32 ± 0.033^{e}	11.72 ± 0.771^{f}	6.87 ± 0.215^{e}
	Lyophilizer	44.40 ± 0.369^{a}	1.86 ± 0.029^{d}	76.53 ± 0.692^{a}	17.63 ± 0.984^{d}
	Oven	32.83 ± 0.250^{f}	1.79 ± 0.040^{d}	54.12 ± 0.243^{c}	17.63 ± 0.771^{d}
	Shade	41.09 ± 0.450^{b}	2.52 ± 0.021^{c}	62.13 ± 0.138^{b}	26.45 ± 0.739^{c}
O. apifera	Fresh	3.52 ± 0.031^{h}	0.12 ± 0.006^{e}	6.87 ± 0.492^{g}	4.52 ± 0.489^{f}
	Lyophilizer	38.59 ± 0.231^{c}	2.31 ± 0.22^{c}	54.36 ± 0.917^{c}	16.82 ± 0.292^{d}
	Oven	34.00 ± 0.415^{e}	6.73 ± 0.040^{a}	35.18 ± 1.01^{e}	41.49 ± 0.609^{a}
	Shade	35.59 ± 0.254^{d}	4.26 ± 0.033^{b}	46.19 ± 1.225^{d}	34.13 ± 0.769^{b}

TE: Trolox equivalent. Letters in rows indicate statistically significant differences ($p < 0.05$).

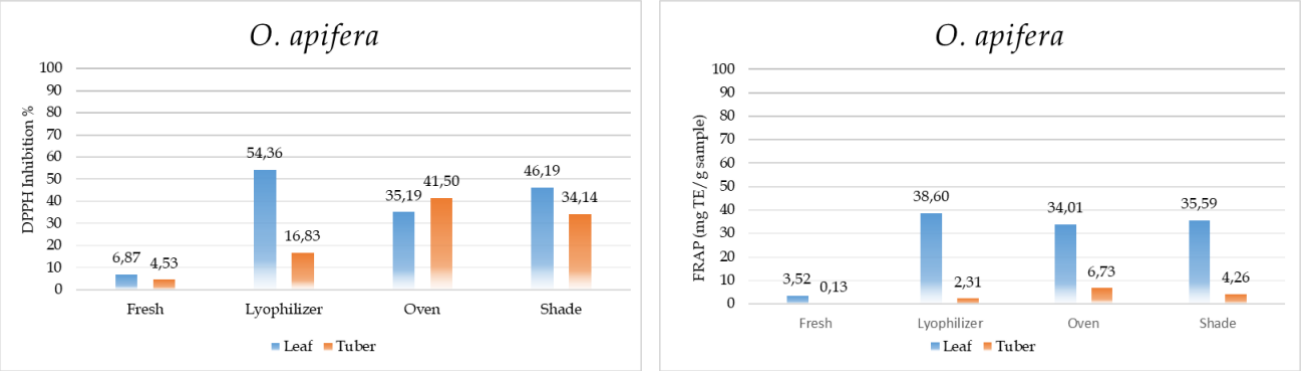

Figure 4. Effect of drying techniques on *O. apifera* and *A. palustris* antioxidant activity

It was observed that the most suitable drying technique for *A. palustris* leaves was lyophilizer and the most suitable drying technique for tubers was shade drying. It was determined that the most suitable drying technique for *O. apifera* leaves was lyophilizer and the most suitable drying technique for tubers was oven drying. The antioxidant results obtained within the scope of our study showed similarities with the research conducted by Stajner et al. [35]. The antioxidant results showed that both DPPH radical scavenging activity and FRAP total reducing power were higher in the aboveground part. In the study conducted by Ertürk et al. (2023), it was stated that good antioxidant activity results depend on the total phenolic content [21]. Our study also shows that orchids obtained in Türkiye and consumed for many years have significant antioxidant activity with different drying methods and such activity is dependent on total phenolic content.

3. CONCLUSION

In the present study, the antioxidant and antibacterial efficiencies, as well as the phenolic composition, of the aqueous extracts derived from the leaves and tubers of *O. apifera* and *A. palustris*, were delineated utilizing High-Performance Liquid Chromatography (HPLC). Based on the findings, nine distinct phenolic compounds were identified within the aqueous extracts of the aforementioned species. The phenolic compounds epicatechin, gallic acid, rutin, and syringic acid were predominantly detected in the HPLC analysis. Rutin and syringic acid were specifically identified in the aqueous extracts of *A. palustris*. Conversely, the aqueous extracts of *O. apifera* exhibited the presence of rutin, syringic acid, epicatechin, and gallic acid. Neither of the aqueous extracts demonstrated any antibacterial efficacy against the tested microorganisms; however, they exhibited remarkably potent antioxidant activities. The results obtained from the DPPH free radical scavenging assay indicated that the highest antioxidant activity in *A. palustris* was recorded in leaves subjected to lyophilization (76.53%), followed by those dried in shade (62.13%) and in an oven (54.12%), respectively. Overall, it was ascertained that the plant exhibited significant activity with

respect to the antioxidant properties investigated. These effects are hypothesized to be attributable to the phenolic constituents present within the plant. In conclusion, both *O. apifera* and *A. palustris* demonstrate potential for application as natural sources of antioxidants. Nevertheless, it would be advantageous to substantiate these activities through further investigations aimed at elucidating their pharmacological applications.

4. MATERIALS AND METHODS

4.1. Plant materials

Plant materials were obtained from the Central Black Sea region of Türkiye, where they are grown commercially. *Anacamptis palustris* and *Ophrys apifera* orchid species grown in Bafra, Samsun, were used as plants materials in this research.

4.2. Plant Extraction

Leaves and tuber parts of the plant were used as fresh and dried by three different techniques: shade, lyophilizer and oven (30-35 °C, 3 days). The samples obtained from the leaves and tubers were ground in a mill and powdered. 0.5 grams of powdered samples were weighed and transferred into the tube. 20 mL of distilled water was added into these tubes, vortexed vigorously and incubated in an incubator shaker at 45°C at 150 rpm for 24 hours. After incubation, it was centrifuged at 2000 g for 10 minutes. Leaf and tuber extracts were filtered using filter paper. The obtained aqueous extracts were aliquoted and stored in the dark at -20°C for use in antioxidant, antibacterial and HPLC analyses [36].

4.3. HPLC Analysis

Polyphenolic compound analysis of *Anacamptis Palustris* and *Ophrys Apifera* aqueous extracts was carried out according to the method of Paje et al. with some modifications [37]using an Agilent brand, 1260 Infinity model HPLC device at Gümüşhane University Central Research Laboratory Application and Research Center. The system consists of G1311C/1260 Quat Pump VL model gradient pump, G1329/1260 ALS model autosampler, G1316A/1260 TCC model column oven and G1315D/1260 DAD VL model detector. A Macherey-nagel, EC 250x4.6 nucleosyl 100-5, 5 µm C18 HD column was used for analysis. The column oven temperature was set to 27 °C. Ultrapure water (phase A), 100% acetonnitrile (phase B), 3% acetic acid/ultrapure water (phase C) and 3% acetic acid + 25% acetonnitrile + ultrapure water (phase D) were used as mobile phase. HPLC gradient profile was 10-20% D/90-80% C (0-10 min.), 20-30% D/80-70% C (10-17 min.), 30-50% D/70-50% C (17-22 min.), 50-80% D/50-20% C (22-32 min.), 20% D/80% C (32-40 min.), 80-20% D/20-0% C/0-80% B (40-45 min.), 20% D/80% B (45-50 min.), 20-10% D/0-90% C/80-0% B (45-50 min.), 10% D/90% C (55-60 min 20 µl of injection volume and a mobile phase flow rate of 0.8 ml/min. Shown in Figure 5 is the HPLC chromatogram of the standard polyphenolic solution. Additionally, analytical validation study was performed for HPLC analysis of polyphenolic compounds and analytical validation parameters are shown in Table 2.

4.4. Antioxidant Activity

4.4.1. Ferric Reducing Antioxidant Power (FRAP) Assay

The milligrams trolox equivalent/gram of plant was used to express the plant extracts' ability to reduce iron. Following a modification of Oyaizu's approach [38], 40 µL of plant extract was combined with 0.2 M phosphate buffer (pH:6.6) and potassium ferricyanate, and the mixture was incubated at 50°C in a dark environment for 20 minutes. After the incubation period, the mixture was placed under water and allowed to cool. After adding 10% TCA (trichloroacetic acid), the liquid was centrifuged for 10 minutes at 2000 g. The distilled water and iron (III) chloride were put to a 96-well microplate containing the supernatants of the centrifuged samples. The finished mixture was allowed to sit at room temperature for five minutes in the dark. A microplane reader was used to detect absorbance at 700 nm following incubation. Every analysis was conducted three times.

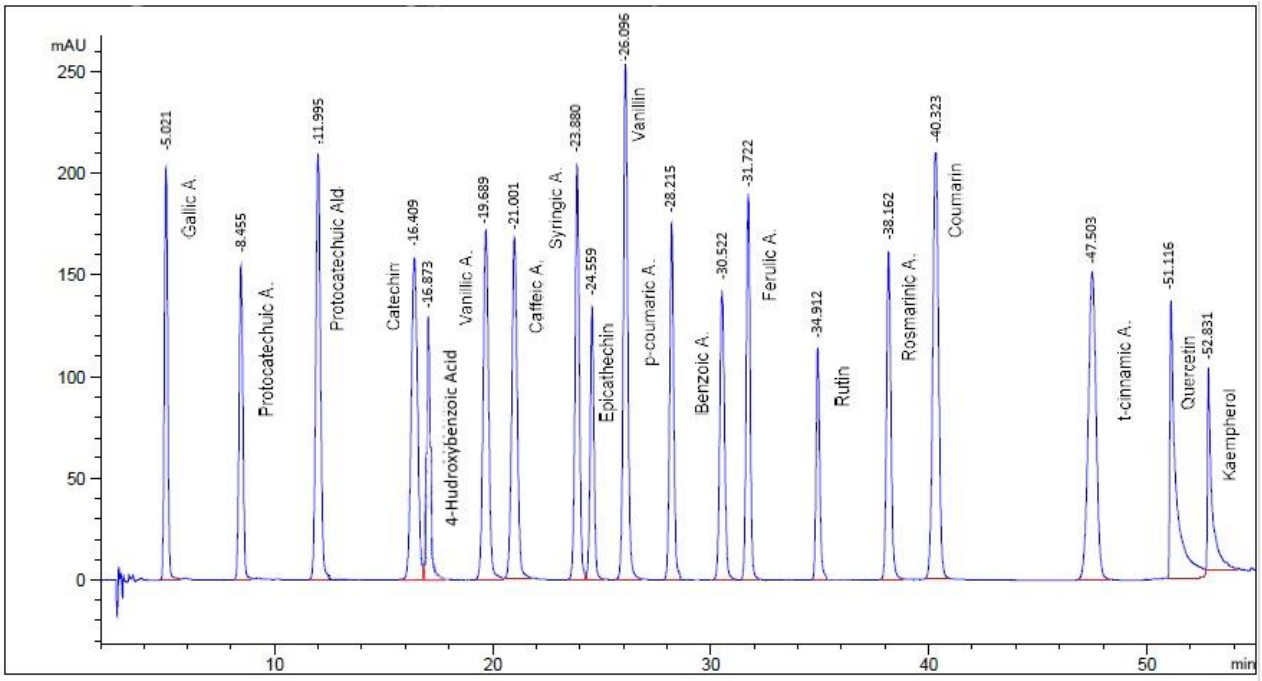

Figure 5. HPLC chromatogram of the polyphenol standard solution

Table 2. Analytical method validation parameters of phenolic compounds analyzed in HPLC

No	Analytes	RT	Correlation	r^2	RSD_R%	RSD_r%	Linearity Range (mg/L)	LOD/LOQ (mg/L)	Recovery(%)
					Interday	Intraday			
1	Gallic Acid	5.021	y=171.892x -16.386	0.995	2.18	1.52	5-100	1.27/4.22	100.0
2	Protocatechuic Acid	8.455	y=- 34.326x -3.408	0.999	1.30	0.63	5-100	0.80/2.65	99.8
3	Protocatechuic Aldehyde	11.995	y=- 164.513x +20.025	0.999	2.42	1.48	5-100	1.02/3.39	99.8
4	Catechin	16.409	y=5.359x -63.413	0.999	1.24	0.81	5-100	1.68/5.48	100.1
5	4-Hydroxybenzoic Acid	16.873	y=- 36.111x -8.048	0.999	1.30	1.02	5-100	2.03/6.78	99.6
6	Vanillic Acid	19.689	y=- 39.531x +9.3444	0.998	2.11	1.09	5-100	1.31/4.36	99.7
7	Caffeic Acid	21.001	y=-78.796x -11.629	0.971	2.42	0.93	5-100	1.25/4.18	99.6
8	Syringic Acid	23.880	y=- 71.521x+6.906	0.998	1.18	0.63	5-100	0.75/2.50	99.7
9	Sesamol	24.155	y= 23.118x-26.735	0.999	2.11	1.25	5-100	0.51/1.71	100.1
10	Epicatechin	24.559	y=-17.582x +5.666	0.999	2.11	1.08	5-100	0.50/1.65	100.2
11	Vanillin	26.096	y=- 95.730x +4.534	0.998	2.18	1.09	5-100	0.41/1.37	99.7
12	P Coumaric Acid	28.215	y= 119.591x -12.267	0.999	2.40	1.16	5-100	0.91/3.04	99.7
13	Benzoic Acid	30.522	y=-9.716x-2.535	0.999	2.40	0.51	5-100	1.41/4.71	100.1
14	Syringaldehyde	31.455	y=- 53.674x +4.475	0.998	2.18	1.16	5-100	0.76/2.54	100.0
15	Ferulic Acid	31.722	y=-68.387x -32.358	0.997	2.18	1.22	5-100	0.77/2.56	99.6
16	Rutin	34.912	y=- 16.562x -2.353	0.998	1.92	0.76	5-100	1.26/4.19	100.2
17	Rosmarinic Acid	38.162	y=- 40.241x -1.551	0.996	1.47	0.62	5-100	0.32/1.07	100.1
18	Coumarin	40.323	y=-101.687x +17.691	0.998	1.47	0.73	5-100	0.47/1.55	99.6
19	t-cinnamic Acid	47.503	y=- 194.844x -25.077	0.998	2.12	1.48	5-100	1.24/4.15	99.7
20	Quercetin	51.116	y=- 27.251x -1.527	0.999	1.94	0.97	5-100	0.71/2.33	100.3
21	Kaempherol	52.831	y= 15.443x-0.862	0.998	2.40	0.57	5-100	0.59/1.97	100.0
22	Crysin	52.969	y=68.444x -1.762	0.957	1.92	0.76	5-100	0.50/1.67	99.7

RT: Retention Time, LOD: Limit of detection, LOQ Limit of Quantitation

4.4.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of plant extracts was determined by modifying the method developed by Yu et al [39]. 125 µL of 0.1 mM DPPH radical was added to 125 µL of plant extract and incubated in the dark at room temperature for 30 min. After incubation, absorbance values

were read on a microplane reader at 517 nm. Results were expressed as % inhibition. Percent inhibition value was calculated from the formula in Equation 1 [40]. Whereas $Abs_{Control}$ was absorbance of DPPH, Abs_{Sample} was absorbance of the sample.

$$\text{Inhibition \%} = ((Abs_{Control} - Abs_{Sample})/Abs_{Control}) \times 100 \quad \textbf{(Equation 1)}$$

4.5. Antibacterial Activity

Antibacterial activity was tested via disk diffusion method with small modifications [41]. Each extract was tested on one Gram (+) positive (*Staphylococcus aureus* ATCC 25923) and two Gram (-) negative (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) bacteria. 100 µl of bacterial suspension was spread thoroughly on agar surface. Then blank disks were impregnated with 20 µl of each extract was placed on the agar surface. Then all agar plates were incubated at 37°C for 24 hours. Antibacterial activity was calculated by measuring the inhibition zone in mm. Standard antibiotic disk was used as control.

4.6. Statistical Analysis

Every experiment was carried out in triplicate. SPSS software was used to analyze the results using a one-way ANOVA (version 25.0), and Duncan's multiple range tests were applied to identify significant differences ($p < 0.05$)

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REFERENCES

[1] Hürkan K, Yüksel MB, Hürkan YK, Demir N. Determination of total phenolic and flavonoid contents, antioxidant and antimicrobial activities of some important salep orchids. Eskişehir Teknik Üniversitesi Bilim ve Teknoloji Dergisi-C Yaşam Bilimleri ve Biyoteknoloji. 2019;8(2):191-202. https://doi.org/10.18036/estubtdc.598852.
[2] Erzurumlu G, Doran İ. Türkiye'de salep orkideleri ve salep kültürü. Harran Tarım ve Gıda Bilimleri Dergisi. 2011;15(1):29-34.
[3] Tekinşen KK. Maraş Salebi. Akademik Gıda. 2006;4(5):28-30.
[4] Durmuşkahya C, Akyol Y, Özdemir C. Ecology, anatomy and morphology of Orchis spitzelii in Turkey. J Environ Biol. 2015;36(Spe):177-184.
[5] Popova E, Kim HH, Saxena PK, Engelmann F, Pritchard HW. Frozen beauty: The cryobiotechnology of orchid diversity. Biotechnol Adv. 2016;34(4):380-403. https://doi.org/10.1016/j.biotechadv.2016.01.001.
[6] Kaki A, Vafaee Y, Khadivi A. Genetic variation of Anacamptis coriophora, Dactylorhiza umbrosa, Himantoglossum affine, Orchis mascula, and Ophrys schulzei in the western parts of Iran. Ind Crop Prod. 2020;156:112854. https://doi.org/10.1016/j.indcrop.2020.112854.
[7] Kurt D, Çalışkan Ö. Doğal Florada Çimlenmiş Sekiz Farklı Salep Orkidesi Türünün İlk Yıl Gelişimlerinin Tanımlanması. Int J Agric Wildlife Sci. 2020;6(2):302-309. https://doi.org/10.24180/ijaws.680153.
[8] Deniz İG, Kömpe YÖ, Harzli I, Aytar EC, Mutlu VA, Uysal Dİ. From seed to flowering tuberous orchid using ex vitro symbiotic seed germination: A breakthrough study with *Anacamptis sancta*. Rhizosphere. 2022;24:100597. https://doi.org/10.1016/j.rhisph.2022.100597.
[9] Cozzolino S, Cafasso D, Pellegrino G, Musacchio A, Widmer A. Fine-scale phylogeographical analysis of Mediterranean *Anacamptis palustris* (Orchidaceae) populations based on chloroplast minisatellite and microsatellite variation. Mol Ecol. 2003;12(10):2783-2792. https://doi.org/10.1046/j.1365-294X.2003.01958.x
[10] Paul-Marian S. *Ophrys apifera* (Orchidaceae) in Transylvanian flora, Romania. Acta Horti Bot Bucurest. 2016;43:31-40. https://doi.org/10.1515/ahbb-2016-0003.
[11] Turkmen N, Gursoy A, Akal C, Unal EM, Keskin E. Evaluation of salep obtained from different wild orchid species of Turkey and their use in Maras type ice cream. J Food Process Preserv. 2021;45(12):e16063. https://doi.org/10.1111/jfpp.16063.

[12] Cocco E, Maccioni D, Sanjust E, Falconieri D, Farris E, Maxia A. Ethnopharmacobotany and diversity of Mediterranean endemic plants in Marmilla subregion, Sardinia, Italy. Plants. 2022;11(22):3165. https://doi.org/10.3390/plants11223165.
[13] Karioti A, Kitsaki CK, Zygouraki S, Ziobora M, Djeddi S, Skaltsa H, Liakopoulos G. Occurrence of flavonoids in Ophrys (Orchidaceae) flower parts. Flora-Morphology, Distribution, Functional Ecology of Plants. 2008;203(7):602-609. https://doi.org/10.1016/j.flora.2007.09.009.
[14] Pin KY. Impact of Drying on Various Quality Characteristics of Herbs, Spices, and Medicinal Plants. Drying of Herbs, Spices, and Medicinal Plants: CRC Press; 2023. p. 53-76. https://doi.org/10.1201/9781003269250.
[15] Yuan J, Hao L-J, Wu G, Wang S, Duan J-a, Xie G-Y, Qin M-J. Effects of drying methods on the phytochemicals contents and antioxidant properties of chrysanthemum flower heads harvested at two developmental stages. J Funct Food. 2015;19:786-795. https://doi.org/10.1016/j.jff.2015.10.008.
[16] Chong CH, Dibagar N, Maulion R, Figiel A. Drying Characteristics of Herbs, Spices, and Medicinal Plants. Drying of Herbs, Spices, and Medicinal Plants: CRC Press; 2023. p. 29-52. https://doi.org/10.1201/9781003269250.
[17] Yougoubo A, Dabire MC, Bationo KR, Ganame A, Noba A, Koala M, Pale E, Roger Nebie CH, Duez , Nacro M. Antioxidants activities, HPTLC and HPLC/MS characterization of some phenolic acids of *Grangea maderaspatana* roots extracts from Burkina Faso. Int J Biol Chem Sci. 2024;18(3):1152-1165. https://doi.org/10.4314/ijbcs.v18i3.33.
[18] Sarmah D, Borah KK, Bora U. Aqueous extracts of biomass ash as an alternative class of Green Solvents for organic transformations: A review update. Sustain Chem Pharm. 2021;24:100551. https://doi.org/10.1016/j.scp.2021.100551.
[19] Boeing JS, Barizão ÉO, e Silva BC, Montanher PF, de Cinque Almeida V, Visentainer JV. Evaluation of solvent effect on the extraction of phenolic compounds and antioxidant capacities from the berries: application of principal component analysis. Chem Cent J. 2014;8:1-9. https://doi.org/10.1186/s13065-014-0048-1.
[20] Van HT, Buı TNH, Maı YH, Nguyen MNH, Pham TV, Doan VD. Chemical Composition and Antibacterial Activities of Ethanol Extract of *Geodorum attenuatum* Griff.(Orchidaceae). Gazi University J Sci. 2020;33(3):613-20. https://doi.org/10.35378/gujs.669681.
[21] Ertürk Ö, Ayvaz MÇ, Çil E, Bağdatlı E. Gas Chromatography-Mass spectrometry analysis and antimicrobial and antioxidant activities of some orchid (Orchidaceae) species growing in Turkey. Brazil Arch Biol Technol. 2023;66:e23210265. https://doi.org/10.1590/1678-4324-2023210265.
[22] Wati RK, De Graaf EF, Bogarín D, Heijungs R, Van Vugt R, Smets EF, Gravendeel B. Antimicrobial activity of necklace orchids is phylogenetically clustered and can be predicted with a biological response method. Front Pharmacol. 2021;11:586345. https://doi.org/10.3389/fphar.2020.586345.
[23] Tanaydın G, Satıl F, Çakılcıoğlu U. An ethnobotanical research on plants used for food purposes in Bigadiç (Balıkesir-Turkey). Türk Doğa ve Fen Dergisi. 2022;11(4):142-147. https://doi.org/10.46810/tdfd.1172635.
[24] Mekonnen AB, Mohammed AS, Tefera AK. Ethnobotanical study of traditional medicinal plants used to treat human and animal diseases in Sedie Muja District, South Gondar, Ethiopia. Evid-Base Complement Altern Med. 2022;2022(1):7328613. https://doi.org/10.1155/2022/7328613.
[25] Emre G, Dogan A, Haznedaroglu MZ, Senkardes I, Ulger M, Satiroglu A, Can Emmez B, Tugay O. An ethnobotanical study of medicinal plants in Mersin (Turkey). Front Pharmacol. 2021;12:664500. https://doi.org/10.3389/fphar.2021.664500.
[26] Sharif A, Shah NA, Rauf A, Hadayat N, Gul A, Nawaz G, Sakhi S, Iqbal M, Khan MR, Shah AA, Azam N, Iftikhar H, Shah SA, Bahadur S, Hussain F, Shuaib M. Ethnomedicinal uses of plants for various diseases in the remote areas of Changa Manga Forest, Pakistan. Braz J Biol. 2022 ;84:e255916. https://doi.org/10.1590/1519-6984.255916.
[27] Ibrahim N, Kebede A. In vitro antibacterial activities of methanol and aqueous leave extracts of selected medicinal plants against human pathogenic bacteria. Saudi J Biol Sci. 2020;27(9):2261-2268. https://doi.org/10.1016/j.sjbs.2020.06.047.
[28] Parekh J, Chanda S. In vitro antimicrobial activity and phytochemical analysis of some Indian medicinal plants. Tur J Biol. 2007;31(1):53-58.
[29] Seck I, Ndoye SF, Kapchoup MVK, Nguemo F, Ciss I, Ba LA, Ba A, Sokhna S, Seck M. Effects of plant extracts and derivatives on cardiac K+, Nav, and Cav channels: a review. Nat Prod Res. 2025;39(5):1213-1240. https://doi.org/10.1080/14786419.2024.2337112.
[30] Kola V, Carvalho IS. Plant extracts as additives in biodegradable films and coatings in active food packaging. Food Biosci. 2023:102860. https://doi.org/10.1016/j.fbio.2023.102860.
[31] Rasheed HA, Rehman A, Karim A, Al-Asmari F, Cui H, Lin L. A comprehensive insight into plant-derived extracts/bioactives: Exploring their antimicrobial mechanisms and potential for high-performance food applications. Food Biosci. 2024:104035. https://doi.org/10.1016/j.fbio.2024.104035.
[32] Hunyadi A. The mechanism (s) of action of antioxidants: From scavenging reactive oxygen/nitrogen species to redox signaling and the generation of bioactive secondary metabolites. Med Res Rev. 2019;39(6):2505-2533. https://doi.org/10.1002/med.21592.
[33] Srinivasulu C, Ramgopal M, Ramanjaneyulu G, Anuradha C, Kumar CS. Syringic acid (SA)–a review of its occurrence, biosynthesis, pharmacological and industrial importance. Biomed Pharmacother. 2018;108:547-557. https://doi.org/10.1016/j.biopha.2018.09.069.

[34] Rotimi DE, Elebiyo TC, Ojo OA. Therapeutic potential of rutin in male infertility: A mini review. J Integ Med. 2023;21(2):130-135. https://doi.org/10.1016/j.joim.2023.01.004.
[35] Štajner D, Popović BM, Kapor A, Boža P, Štajner M. Antioxidant and scavenging capacity of *Anacamptis pyrimidalis* L.–pyrimidal orchid from Vojvodina. Phytother Res. 2010;24(5):759-763. https://doi.org/10.1002/ptr.3041.
[36] Turan I, Canbolat D, Demir S, Kerimoglu G, Colak F, Turkmen Alemdar N, Mentese A, Aliyazicioglu Y. The ameliorative effect of Primula vulgaris on cisplatin-induced nephrotoxicity in rats and quantification of its phenolic components using LC-ESI-MS/MS. Saudi Pharm J. 2023 ;31(9):101730. https://doi.org/10.1016/j.jsps.2023.101730.
[37] Paje LA, Choi J, Lee HD, Kim J, Yu AR, Bae MJ, Geraldino PJL, Lee S. Phenolic acids and flavonoids from Salvia plebeia and HPLC-UV profiling of four Salvia species. Heliyon. 2022;8(3):e09046. https://doi.org/10.1016/j.heliyon.2022.e09046.
[38] Oyaızu M. Studies on product of browning reaction prepared from glucose amine. Japan J Nutr. 1986;44:307-315. http://dx.doi.org/10.5264/eiyogakuzashi.44.307.
[39] Yu L, Haley S, Perret J, Harris M, Wilson J, Qian M. Free radical scavenging properties of wheat extracts. J Agric Food Chem. 2002;50(6):1619-1624. https://doi.org/10.1021/jf010964p.
[40] Canbolat D, Turan İ, Küpeli YE, Kılınç S, Piliç S. Artvin Şavşat Yöresi Propolisinin Farklı Sıcaklıklardaki PBS'li Ekstraktlarının Antioksidan Özelliklerinin ve Eritrosit Hemoliz İnhibisyonu Üzerine Etkisinin Araştırılması. Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi. 2021;24(3):464-472. https://doi.org/10.18016/ksutarimdoga.vi.765838.
[41] Gedikoğlu A, Sökmen M, Çivit A. Evaluation of *Thymus vulgaris* and *Thymbra spicata* essential oils and plant extracts for chemical composition, antioxidant, and antimicrobial properties. Food Sci Nutr. 2019;7(5):1704-1714. https://doi.org/10.1002/fsn3.1007