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DETERMINATION OF TOTAL PHENOLIC AND FLAVONOID CONTENTS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF SOME IMPORTANT SALEP ORCHIDS

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ABSTRACT

In this study we evaluated the secondary metabolites, total phenolic (TPC) and flavonoid contents (TFC), antioxidant and antimicrobial activities of salep orchids, *Anacamptis morio*, *Anacamptis pyramidalis*, *Neotinea tridentata*, *Ophrys mammosa*, *Ophrys lutea*, and *Ophrys speculum*. DPPH free radical scavenging assay was used to determine the antioxidant activities of *n*-hexane, chloroform, methanol and water extracts of the plants. The antimicrobial activities were also determined by the Broth micro-dilution method. The extracts were studied for antimicrobial activity by the Minimum Inhibitory Concentration (MIC) approach against seven clinical pathogenic bacteria and two fungi. Phytochemical screening revealed that the presences of coumarins, flavonoids, flavanones, cardiac glycosides, proteins and quinones. The extracts had variable TPC and TFC, with values of 4.46 ± 0.19–45.83 ± 1.86 mg gallic acid equivalent/g dry weight and 0.67 ± 0.04–8.64 ± 0.37 mg quercetin equivalent/g dry weight respectively. *O. speculum* had the highest (35.12%) antioxidant activity, followed by *O. mammosa* (33.17%). Chloroform extracts of all species showed significant antioxidant and antimicrobial activity. These bioactivities of the chloroform extracts were positively associated with the total phenolic and flavonoid contents. The MIC concentrations ranged from 0.156–20 mg/mL. The present investigation shows that the extracts of these species, especially chloroform extracts, could be used as potential antioxidant and antimicrobial sources.

Keywords: Salep orchids, Antioxidant activity, DPPH• assay, Antimicrobial activity, Minimum inhibitory concentration

BAZI ÖNEMLİ SALEP ORKİDESİ TÜRLERİNİN TOPLAM FENOLİK VE FLAVONOİT İÇERİKLERİNİN, ANTİOKSİDAN VE ANTİMİKROBİYAL AKTİVİTELERİNİN BELİRLENMESİ

ÖZET

Bu çalışmada; *Anacamptis morio*, *Anacamptis pyramidalis*, *Neotinea tridentata*, *Ophrys mammosa*, *Ophrys lutea* ve *Ophrys speculum* salep orkidelerinin sekonder metabolitleri, toplam fenolik (TPC) ve flavonoit bileşikleri (TFC), antioksidan ve antimikrobiyal aktiviteleri ölçülmüştür. DPPH serbest radikal temizleme yöntemi ile bitkilerin *n*-hekzan, kloroform, methanol ve su özütlerinde antioksidan aktiviteler belirlenmiştir. Antimikrobiyal aktiviteler Broth mikrodilüsyon yöntemi ile belirlenmiştir. Özütler yedi klinik patojen bakteriye ve iki fungusa karşı taranmıştır. Fitokimyasal taramada kumarinler, flavanoitler, flavanonlar, kardiyak glikozitler, proteinler ve kinonlar bulunduğunu ortaya çıkarılmıştır. Özütlerin değişken TPC ve TFC değerlerine sahip olduğu gözlenmiştir (TPC 4.46 ± 0.19–45.83 ± 1.86 mg gallik asit eşdeğer/g kuru ağırlık ve TFC 0.67 ± 0.04–8.64 ± 0.37 mg kuersetin eşdeğer/g kuru ağırlık). Çalışmada *O. speculum* türünün %35.12 ile en yüksek antioksidan aktiviteye sahip olduğu ve onu %33.17 ile *O. mammosa* türünün takip ettiği belirlenmiştir. Bütün türlerde kloroform özütlerinin en yüksek antioksidan ve antimikrobiyal aktiviteye sahip olduğu görülmüştür. Kloroform özütlerinin bu biyoaktiviteleri toplam fenolik ve flavanoit bileşikleri ile pozitif olarak ilişkilidir. MİK konsantrasyonları 0.156-20 mg/mL oranındadır. Bu çalışma, kullanılan salep orkidelerinin özellikle kloroform özütlerinin, potansiyel antioksidan ve antimikrobiyal kaynakları olabileceğini göstermektedir.

Anahtar Kelimeler: Salep orkideleri, Antioksidan aktivite, DPPH• analizi, Antimikrobiyal aktivite, Minimum inhibisyon konsantrasyonu

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1. INTRODUCTION

Orchids are one of the largest flowering-plant family [1]. They have been used as healing extracts, perfumes and food additives in many different cultures such as Oriental, European, American, Australian and African communities for centuries [2]. Turkey is one of the most orchid-rich countries in Europe with 170 terrestrial orchids [3]. Tubers of about 120 orchid species belonging to *Aceras*, *Anacamptis*, *Barlia*, *Comperia*, *Dactylorhiza*, *Himantoglossum*, *Neotinea*, *Ophrys*, *Orchis*, and *Serapias* genera are used to produce hot drink salep in Turkey [4]. Salep is a very popular and traditional nutritious hot-drink in the Asia-Minor and Arabic countries. It is made from dried tubers of several terrestrial orchid species. *A. morio*, *A. pyramidalis*, *N. tridentata*, *O. lutea*, *O. mammosa*, and *O. speculum* orchids are tuberous orchids that naturally grow in Turkey and used to made salep [4]. The stimulant effect of salep on the generative power has been known since Dioscorides and the Arabian [5]. Moreover, salep is also used as a stabiliser-additive in Maras ice cream which is special for Turkey [3,6,7]. Aphrodisiac, anti-inflammatory, antidiarrheal, appetising and expectorant effects of salep are also known [2,6].

Studies on orchid chemicals have shown the main compounds are carbohydrates, alkaloids, flavonoids, glycosides, bibenzyl derivatives, alkaloids and terpenoids [8, 9]. Currently, there are fifty orchid species used in traditional Chinese medicine [10]. Orchids are also used as Ayurvedic medicines in India [11]. Moreover, *Malaxis muscifera* (Lindley) Kuntze orchid is included in the Astavarga eight medicinal plants list [12]. Pharmacologically, orchids have diuretic, anti-inflammatory, anti-carcinogenic, hypoglycemic, anti-rheumatic and neuroprotective effects [13].

The plants are a natural antioxidant source because of their secondary metabolites. Natural substances like phenolics, flavonoids, which are secondary plant metabolites, also exhibit a wide range of biological and pharmacological properties, including antioxidant, antimicrobial, anti-inflammatory, antimutagenic effects etc. [14]. Higher plants synthesize approximately 8.000 known phenolic compounds. Many phenolic compounds have important roles in plants as defenses against herbivores and pathogens. Polyphenols are proven to have bactericidal activities in the treatment of bacterial infections [15]. Flavonoids act as possible neuroprotective agents in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [16,17]. Orchids constitute an effective source of antioxidant natural substances [18,19,20]. Antioxidant substances protect the body against free radicals, especially reactive oxygen species (ROS), that can cause cancer, atherosclerosis, diabetes mellitus, cardiovascular diseases, ageing, neurodegenerative diseases, and inflammatory diseases [21,22]. Scientists are trying to find natural antioxidants, after they discovered that synthetic antioxidants pose health risks.

In recent years, there have been a number of studies investigating the antioxidant and antimicrobial potential of plants. In this study we aimed to investigate: 1) Various phytochemical compounds, 2) *in vitro* antioxidant activity using $DPPH^{\bullet}$ assay and antimicrobial effects by the Broth micro-dilution method against various pathogenic bacteria and fungi strains of salep orchids *A. morio*, *A. pyramidalis*, *N. tridentata*, *O. mammosa*, *O. lutea* and *O. speculum*.

2. MATERIALS AND METHODS

2.1. Chemicals

Gallic acid ($C_7H_6O_5$), 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), sodium hydroxide (NaOH), *n*-hexane (C_6H_{14}), tetraoxosulphate (VI) acid (H_2SO_4), chloroform ($CHCl_3$), ethanol (C_2H_5OH), methanol (CH_3OH) and ferric chloride were obtained from Sigma-Aldrich (Germany). The other chemicals and reagents were purchased from Merck (Bangalore). All the reagents were analytical grade.

2.2. Plant Materials and Preparation of Extracts

All plant materials, *Anacamptis morio* (L.) R. M. Bateman, Pridgeon & M. W. Chase, *Anacamptis pyramidalis* Rich., *Neotinea tridentata* (Scop.) R. M. Bateman, Pridgeon & M. W. Chase, *Ophrys mammosa* Desf., *Ophrys lutea* Cav., and *Ophrys speculum* Link., were collected from Çanakkale (Turkey) during their blooming periods (March to May) in 2015. The voucher specimen of the orchid species preserved at the herbarium of Biology Department, Çanakkale Onsekiz Mart University, Çanakkale, Turkey.

The plants were cleaned by distilled water and ground in mortar by liquid nitrogen. Then plant powder (15 g) was packed into a Soxhlet apparatus and extracted with 300 mL *n*-hexane, chloroform, methanol and distilled water, respectively for 12 hours. The extracts were filtered through Whatman filter paper No. 1, and this was followed by a concentration using a rotary evaporator (Spektral, Heidolph, Laborota 4001) under pressure that was reduced. Then, the crude extracts were kept in sterile bottles at 4 ºC until further analyses.

2.3. Preliminary Qualitative Analysis of Secondary Phytochemicals

Each orchid extract was evaluated for preliminary screening of secondary phytochemicals such as, anthocyanins, cardiac glycosides, coumarins, flavanones, flavonoids, proteins and quinones using established protocols [23, 24, 25] as follows.

Anthocyanins
Ten percent NaOH was added to the plant extract, blue colour indicates the presence of anthocyanins.

Cardiac glycosides
Three ml of each glacial acetic acid, ferric chloride and H_2SO_4 were added to the plant extract. Green colour indicates the presence of cardiac glycosides.

Coumarins
One to one (v/v) 10% of NaOH was added to the plant extract. Yellow colour indicates the presence of coumarins.

Flavanones
Ten percent of few drops of NaOH was added to the 3 ml plant extract. Yellow colour indicates the presence of flavanones.

Flavonoids
Three ml of plant extract was dissolved in 50% of methanol, warmed up to 40°C, added a piece of magnesium ribbon and 1ml of HCl. Red or yellow colour indicates the presence of flavonoids.

Proteins
One ml of 40% NaOH solution and 2ml of 1% CuSO4 were added to the 3 ml of plant extract. Violet colour indicates the presence of proteins.

Quinones
One ml of conc.H2SO4 were added to the 1ml of plant extract. Red colour indicates the presence of quinones.

The results are expressed as (+) for the presence and (-) for the absence of phytochemicals (Table 1).

2.4. Total Phenolic Content

The total phenolic content of the orchid extracts was determined by the Folin-Ciocalteu method [26]. In brief, 0.1 mL of extract solution, 4.5 mL distilled water and 0.1 mL of Folin-Ciocalteu reagent were added and the contents mixed thoroughly. After 3 minutes, 1 mL of 2% Na_2CO_3 was added, and then the mixture was allowed to stand for 2 hours at room temperature in the dark. Then the absorbance of the mixture was measured at 760 nm using a UV spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). The results were expressed as mg of gallic acid equivalent per g of dry weight (mg GAE/g DW) from a calibration curve with gallic acid. All samples were analysed in three replicates and the results were averaged.

2.5. Total Flavonoid Content

The total flavonoid content of the orchid extracts was determined by using the method as described by [27]. 0.1 mL of extract solution, 0.1 mL of 10% $Al(NO_3)_3 . 9H_2O$, 0.1 mL of 1M $C_2H_3KO_2$ and 5.2 mL of ethanol were mixed. After 40 minutes of incubation at room temperature, the absorbance at 415 nm was measured using a UV spectrophotometer. The results were expressed as mg quercetin equivalent per g of dry weight (mg QuE/g DW) from a calibration curve with quercetin. Measurements were done in triplicates and the results were averaged.

2.6. Antioxidant Activity

The antioxidant activity of the extracts was determined using DPPH free radical scavenging assay [28]. Extracts (0.1 mL; 200, 400, 600, 800 and 1000 µg/mL) were added to 1.9 mL of methanol and then 0.5 mL of 0.2 mM solution of DPPH$^{\bullet}$ in methanol solution was added. The mixture was shaken vigorously and maintained in the dark at room temperature for 30 minutes. Then the absorbance was determined against a blank at 517 nm. Butylated hydroxy-toluene (BHT) was used as a positive control. Samples were analysed in triplicate. The percentage inhibition of DPPH free radical was calculated from: DPPH$^{\bullet}$ scavenging activity (%) = [(absorbance of control – absorbance of sample)/(absorbance of control)] × 100.

2.7. Antimicrobial Activity

In vitro antimicrobial activity was examined for various extracts of the orchid species. Nine pathogenic microorganism strains were used: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *E. coli* (NRRL B-3704), *Pseudomonas aeruginosa* (ATCC 10145), *Proteus vulgaris* (ATCC 13315), *Candida albicans* (ATCC 60193) and *C. tropicalis* (ATCC 13803). Stock cultures were maintained at 4 ºC on Mueller-Hinton Agar (MHA), and Sabouraud Dextrose Agar (SDA) plates were used for bacteria and fungi respectively.

The antimicrobial activity of the plant extracts was determined by Minimum Inhibitory Concentration (MIC) assay. MIC was carried out according to the methods of Clinical and Laboratory Standards Institute [29, 30]. MIC values of orchid extracts were assessed by the Broth micro-dilution method using the 96-well plates. An inoculum of the microorganism was prepared from 18-hour fresh cultures. Suspensions were adjusted with a turbidity equivalent to McFarland No. 0.5 standard, and thus, inoculums were achieved to ~1.5×10^8 CFU/mL. Plant extracts prepared in dimethyl sulfoxide (DMSO) were diluted with Mueller Hinton Broth (MHB) and RPMI-1640 for antibacterial and antifungal analyses respectively. Total of 100 µL culture suspension was added to each well containing 100 µL of extracts in concentration range between 20 to 0.156 mg/mL. And then the plates were incubated aerobically at 35-37 ºC for 24 hours. Gentamycin and fluconazole served as positive controls for bacteria and fungi while DMSO served as negative control.

2.8. Statistical Analysis

All assays were carried out in triplicate, and their results were expressed as mean ± standard deviation. The data were subjected to Kruskal Wallis-H test with significant difference determined at a confidence level of $p < 0.05$. Statistical analyses were performed with the SPSS statistical software (SPSS v.21).

3. RESULTS

3.1. Secondary Metabolites Analyses

Preliminary phytochemical screening results of the extracts are shown in Table 1. The phytochemical analysis showed that *A. morio*, *A. pyramidalis*, *N. tridentata*, *O. mammosa*, *O. lutea* and *O. speculum* plant extracts contain a mixture of phytochemicals as cardiac glycosides, phenolic compounds, flavonoids, coumarins, flavanones, proteins and quinones.

Table 1. Phytochemical screening of various extracts of tested orchids

Species	Extracts	Tested Phytochemical Compounds						
		Anthocyanins	Cardiac glycosides	Coumarins	Flavanones	Flavanoids	Proteins	Quinones
A. morio	H	-	-	+	+	+	+	-
	C	-	-	+	+	+	+	-
	M	-	-	+	+	+	+	+
	W	-	-	+	+	+	+	+
A. pyramidalis	H	-	+	+	+	+	+	-
	C	-	+	+	+	+	+	-
	M	-	-	+	+	+	+	+
	W	-	-	+	+	+	+	+
N. tridentata	H	-	+	+	+	+	+	-
	C	-	+	+	+	+	+	-
	M	-	-	+	+	+	+	+
	W	-	-	+	+	+	+	+
O. mammosa	H	-	+	+	+	+	+	-
	C	-	+	+	+	+	+	-
	M	-	-	+	+	+	+	+
	W	-	-	+	+	+	+	+
O. lutea	H	-	+	+	+	+	+	-
	C	-	+	+	+	+	+	-
	M	-	-	+	+	+	+	+
	W	-	-	+	+	+	+	+
O. speculum	H	-	+	+	+	+	+	-
	C	-	+	+	+	+	+	-
	M	-	-	+	+	+	+	+
	W	-	-	+	+	+	+	+

H: *n*-Hexane, C: Chloroform, M: Methanol, W: Water.
+: present; -: absent.

3.2. Total Phenolic and Flavonoid Contents

The total phenolic and flavonoid contents of the extracts are shown in Table 2. The total phenolic contents of the extracts, expressed as mg of gallic acid equivalents (GAE), varied from 4.46 ± 0.19 mg GAE/g for the methanol extract of *N. tridentata* to 45.83 ± 1.86 mg GAE/g the chloroform extract of *O.*

speculum. The content of total flavonoid is expressed as mg of quercetin equivalent (QuE) per g of dry weight that ranged from 0.67 ± 0.04 to 8.64 ± 0.37. The total flavonoid content results were entirely synchronous with those of the total phenolic. The chloroform extracts of all species had illustrated the highest total content of phenolic and flavonoid, whereas the content obtained with *n*-hexane, methanol and water were much lower ($p < 0.05$) (Table 2).

Table 2. The total phenolic and flavonoid contents of tested orchid species.

		TPC	TFC
Species	**Extracts**	**mg GAE/g DW**	**mg QuE/g DW**
A. morio	*n*-Hexane	8.33 ± 0.33^{h}	1.55 ± 0.08^{f}
	Chloroform	20.83 ± 0.36^{d}	3.98 ± 0.21^{c}
	Methanol	11.31 ± 0.79^{g}	1.87 ± 0.03^{e}
	Water	11.31 ± 0.43^{g}	1.98 ± 0.05^{e}
A. pyramidalis	*n*-Hexane	13.10 ± 0.66^{f}	2.03 ± 0.02^{e}
	Chloroform	26.19 ± 0.57^{c}	4.06 ± 0.01^{c}
	Methanol	9.52 ± 0.32^{h}	1.67 ± 0.03^{f}
	Water	$18.75 \pm 0.89^{d,e}$	3.78 ± 0.06^{c}
N. tridentata	*n*-Hexane	13.4 ± 0.55^{f}	2.61 ± 0.15^{e}
	Chloroform	19.64 ± 0.68^{d}	$4.04 \pm 0.32^{b,c}$
	Methanol	4.46 ± 0.19^{j}	0.67 ± 0.04^{g}
	Water	6.55 ± 0.07^{i}	1.09 ± 0.06^{g}
O. mammosa	*n*-Hexane	6.84 ± 0.49^{i}	0.95 ± 0.09^{g}
	Chloroform	43.45 ± 1.37^{a}	7.92 ± 0.14^{a}
	Methanol	37.20 ± 0.51^{b}	5.04 ± 0.23^{b}
	Water	10.71 ± 0.90^{g}	1.51 ± 0.11^{f}
O. lutea	*n*-Hexane	$14.58 \pm 0.87^{e,f}$	2.33 ± 0.09^{e}
	Chloroform	35.71 ± 0.90^{b}	4.41 ± 0.13^{b}
	Methanol	13.10 ± 0.86^{f}	1.81 ± 0.07^{e}
	Water	$13.99 \pm 0.91^{e,f}$	2.09 ± 0.05^{e}
O. speculum	*n*-Hexane	21.73 ± 0.77^{d}	3.17 ± 0.28^{d}
	Chloroform	45.83 ± 1.86^{a}	8.64 ± 0.37^{a}
	Methanol	16.37 ± 0.26^{e}	2.34 ± 0.05^{e}
	Water	5.36 ± 0.12^{j}	0.82 ± 0.03^{g}

TPC: Total phenolic content; TFC: Total flavonoid content; GAE: Gallic acid equivalent; DW: Dry weight; QuE: Quercetin equivalent. Value ± SD

*Different letters in the columns indicate significant differences between extracts within TPC and TFC. Means separated by Kruskal Wallis-H test ($p < 0.05$).

3.3. Antioxidant Activity

Antioxidant activities of the orchid extracts have been tested by DPPH radical procedure using butylated hydroxytoluene (BHT) as a reference standard. The tested doses ranged from 20-100 µg. The results are showed in Table 3. The best DPPH$^{\bullet}$ scavenging activity was observed in chloroform extract of *O.*

speculum with 35.12 ± 0.58% at 100 µg. Generally, chloroform extracts had the highest percentage of antioxidant activity. In addition, antioxidant activity of the extracts increased with the dose (Table 3).

Table 3. DPPH free radical scavenging activity for tested orchid species

		DPPH˙ Scavenging Activity (%)				
		Doses (µg)				
		20	**40**	**60**	**80**	**100**
Positive control	BHT	$17.60 \pm 0.10^{a,E}$	$20.21 \pm 0.07^{a,D}$	$27.39 \pm 0.13^{a,C}$	$36.18 \pm 0.07^{a,B}$	$48.68 \pm 0.22^{a,A}$
A. morio	H	$6.34 \pm 0.04^{d,B}$	$7.42 \pm 0.04^{e,B}$	$9.26 \pm 0.19^{e,f,A}$	$9.67 \pm 0.11^{g,A}$	$10.29 \pm 0.08^{h,A}$
	C	$6.77 \pm 0.08^{c,C}$	$8.49 \pm 0.11^{d,B}$	$11.37 \pm 0.04^{e,A,B}$	$12.51 \pm 0.08^{f,A}$	$13.97 \pm 0.11^{g,A}$
	M	$5.26 \pm 0.11^{d,B}$	$6.84 \pm 0.04^{e,A,B}$	$8.42 \pm 0.08^{f,A}$	$8.93 \pm 0.04^{h,A}$	$9.45 \pm 0.11^{h,ı,A}$
	W	$4.91 \pm 0.11^{d,e,B}$	$6.56 \pm 0.15^{e,A}$	$7.23 \pm 0.18^{f,A}$	$7.63 \pm 0.08^{h,A}$	$7.71 \pm 0.11^{ı,A}$
A. pyramidalis	H	$5.65 \pm 0.08^{d,C}$	$7.01 \pm 0.11^{e,C}$	$9.74 \pm 0.15^{e,B}$	$12.78 \pm 0.14^{f,A}$	$14.41 \pm 0.08^{g,A}$
	C	$6.68 \pm 0.21^{c,d,C}$	$10.96 \pm 0.15^{c,B}$	$12.61 \pm 0.04^{d,B}$	$14.02 \pm 0.11^{e,A}$	$16.54 \pm 0.11^{f,A}$
	M	$7.44 \pm 0.11^{c,B}$	$8.64 \pm 0.04^{d,A}$	$9.43 \pm 0.04^{e,A}$	$9.91 \pm 0.07^{g,A}$	$10.24 \pm 0.11^{h,A}$
	W	$8.14 \pm 0.11^{c,B}$	$9.45 \pm 0.04^{d,A}$	$10.07 \pm 0.04^{e,A}$	$10.89 \pm 0.04^{g,A}$	$11.70 \pm 0.07^{h,A}$
N. tridentata	H	$6.73 \pm 0.14^{c,d,C}$	$11.24 \pm 0.05^{c,B}$	$12.09 \pm 0.08^{d,B}$	$13.42 \pm 0.05^{f,A}$	$15.11 \pm 0.26^{f,A}$
	C	$6.98 \pm 0.33^{c,C}$	$11.83 \pm 0.17^{c,B}$	$13.10 \pm 0.52^{d,A,B}$	$14.19 \pm 0.07^{e,A}$	$15.93 \pm 0.21^{f,A}$
	M	$3.71 \pm 0.19^{e,C}$	$7.51 \pm 0.13^{d,e,B}$	$8.18 \pm 0.32^{f,A}$	$8.91 \pm 0.24^{g,h,A}$	$10.11 \pm 0.04^{h,A}$
	W	$2.66 \pm 0.20^{f,C}$	$6.29 \pm 0.19^{e,A}$	$7.13 \pm 0.13^{f,A}$	$7.34 \pm 0.35^{h,A}$	$8.20 \pm 0.21^{ı,A}$
O. mammosa	H	$7.53 \pm 0.42^{c,E}$	$10.30 \pm 0.44^{c,D}$	$16.65 \pm 0.32^{c,C}$	$22.74 \pm 0.16^{c,B}$	$27.14 \pm 0.50^{c,A}$
	C	$9.50 \pm 0.09^{b,E}$	$16.17 \pm 0.94^{b,D}$	$21.48 \pm 0.84^{b,C}$	$26.79 \pm 0.31^{b,B}$	$33.17 \pm 0.39^{b,A}$
	M	$4.35 \pm 0.28^{e,D}$	$11.29 \pm 0.58^{c,C}$	$15.69 \pm 0.35^{c,B}$	$17.88 \pm 0.12^{d,A}$	$19.94 \pm 0.08^{e,A}$
	W	$5.87 \pm 0.33^{d,D}$	$9.85 \pm 0.14^{c,d,C}$	$11.00 \pm 0.09^{e,C}$	$15.40 \pm 0.09^{e,B}$	$23.25 \pm 0.05^{d,A}$
O. lutea	H	$6.27 \pm 0.19^{d,B}$	$6.76 \pm 0.30^{e,A,B}$	$7.86 \pm 0.06^{f,A}$	$8.20 \pm 0.16^{h,A}$	$8.85 \pm 0.10^{ı,A}$
	C	$3.30 \pm 0.10^{e,C}$	$10.32 \pm 0.16^{c,B}$	$11.10 \pm 0.25^{e,B}$	$14.06 \pm 0.10^{e,A}$	$14.83 \pm 0.20^{f,A}$
	M	$6.88 \pm 0.10^{c,C}$	$7.69 \pm 0.16^{d,C}$	$10.36 \pm 0.13^{e,B}$	$12.03 \pm 0.13^{f,B}$	$15.95 \pm 0.04^{f,A}$
	W	$4.66 \pm 0.16^{e,C}$	$8.45 \pm 0.16^{d,B}$	$10.80 \pm 0.16^{e,B}$	$13.18 \pm 0.04^{f,A}$	$14.13 \pm 0.07^{g,A}$
O. speculum	H	$7.50 \pm 0.12^{c,D}$	$10.76 \pm 0.12^{c,C,D}$	$13.50 \pm 0.89^{d,C}$	$18.79 \pm 0.33^{d,B}$	$23.22 \pm 0.16^{d,A}$
	C	$10.70 \pm 0.17^{b,E}$	$17.83 \pm 0.24^{a,D}$	$22.52 \pm 0.49^{b,C}$	$27.60 \pm 0.33^{b,B}$	$35.12 \pm 0.58^{b,A}$
	M	$9.77 \pm 0.28^{b,C}$	$16.17 \pm 0.08^{b,B}$	$16.55 \pm 0.17^{c,B}$	$19.11 \pm 0.39^{d,A}$	$20.87 \pm 0.12^{d,e,A}$
	W	$4.96 \pm 0.08^{d,C}$	$8.33 \pm 0.08^{d,B}$	$10.92 \pm 0.41^{e,A,B}$	$11.08 \pm 0.09^{g,A}$	$13.50 \pm 0.09^{g,A}$

H: *n*-Hexane extract, C: Chloroform extract, M: Methanol extract, W: Water extract. Value ± SD.
* Different lowercase letters in the columns indicate significant differences between tested extracts according to Kruskal Wallis-H test ($p < 0.05$).
**Different uppercase letters in the lines indicate significant differences between doses according to Kruskal Wallis-H test ($p < 0.05$).

3.4. Antimicrobial Activity

The antimicrobial activities of the plants assayed against some human pathogen Gram-positive (*S. aureus*, *E. faecalis*, *B. subtilis*) and Gram-negative (*E. coli*, *P. aeruginosa*, *P. vulgaris*) bacteria and fungi (*C. albicans*, *C. tropicalis*). Antimicrobial activity results are shown in Table 4. The orchid extracts showed a broad spectrum (0.156 – 20 mg/mL) of antimicrobial activity against tested microorganisms.

P. aeruginosa was found to be the most resistant microorganism against orchid extracts. *A. morio n*-hexane extract and *A. pyramidalis* methanol and water extracts were the most effective plant extracts and showed bacteriostatic activity against *P. vulgaris* with 0.156 mg/mL MIC value. *N. tridentata* extracts (except *n*-hexane extract) and *O. speculum* chloroform extract showed strong antifungal inhibition against *C. albicans* at a dose of 0.312 mg/mL (Table 4).

Table 4. Minimum inhibitory concentration (MIC) values of the tested orchids

		MIC (mg/mL)								
		Bacteria							Fungi	
		Gram (+)			Gram (-)					
		Staphylococcus aureus (ATCC 25923)	*Enterococcus faecalis (ATCC 29212)*	*Bacillus subtilis* (ATCC 6633)	*Escherichia coli* (ATCC 25922)	*Escherichia coli* (NRLL B-3704)	*Pseudomonas aeruginosa* (ATCC 10145)	*Proteus vulgaris* (ATCC 13315)	*Candida albicans* (ATCC 60193)	*Candida tropicalis* (ATCC 13803)
A. morio	H	5	1.25	0.312	5	2.5	2.5	0.156	1.25	1.25
	C	2.5	1.25	1.25	2.5	1.25	2.5	0.625	2.5	2.5
	M	5	10	10	5	5	5	0.312	5	5
	W	5	5	10	5	5	5	0.312	2.5	5
A. pyramidalis	H	2.5	1.25	0.312	2.5	5	2.5	1.25	5	2.5
	C	2.5	1.25	1.25	2.5	5	2.5	2.5	2.5	2.5
	M	5	10	10	10	5	10	0.156	5	5
	W	5	10	10	5	5	5	0.156	5	2.5
N. tridentata	H	1.25	20	5	20	20	-	10	10	10
	C	20	20	2.5	10	20	20	20	0.312	10
	M	10	20	10	20	-	-	20	0.312	10
	W	20	20	10	20	20	20	20	0.312	10
O. mammosa	H	20	10	2.5	10	20	-	20	20	5
	C	1.25	20	1.25	20	20	-	-	1.25	5
	M	5	20	5	20	10	20	10	0.625	10
	W	10	20	10	20	20	20	10	0.625	10
O. lutea	H	2.5	0.625	0.312	1.25	2.5	5	0.312	1.25	1.25
	C	2.5	0.312	0.625	1.25	2.5	2.5	0.312	2.5	2.5
	M	5	10	5	5	5	5	0.312	2.5	5
	W	5	5	10	5	5	5	0.625	2.5	2.5
O. speculum	H	10	2.5	2.5	-	10	-	-	2.5	2.5
	C	1.25	1.25	1.25	10	5	10	10	0.312	2.5
	M	10	20	5	20	10	20	10	0.625	5
	W	20	20	10	20	20	20	20	0.625	10
Positive control	G	$1.95.10^{-3}$	16.10^{-3}	$0.98.10^{-3}$	$7.8.10^{-3}$	62.10^{-3}	$1.95.10^{-3}$	$3.9.10^{-3}$	NT	NT
	F	NT	NT	NT	NT	NT	NT	NT	$7.8.10^{-3}$	$1.95.10^{-3}$

H: *n*-Hexane extract, C: Chloroform extract, M: Methanol extract, W: Water extract, G: Gentamycin, F: Fluconazole. -: No inhibition; NT: Not tested.

4. DISCUSSION

Orchids have been used in beverages, healing extracts, perfumes for their healing features and wealthy contents for centuries. Due to the broad variety of salep orchids in nature, especially in the Mediterranean Region, contents of the salep orchids (or potential salep orchids) must be evaluated in detail. In this study, we investigated some frequently used salep orchids in the Mediterranean Region, *A. morio*, *A. pyramidalis*, *N. tridentata*, *O. mammosa*, *O. lutea*, and *O. speculum*, for their secondary metabolites, total phenolic and flavonoid contents, antioxidant and antimicrobial activities. We also analysed the antimicrobial activities of the extracts by the Broth micro-dilution method approach against seven clinical pathogenic bacteria and two fungi. Our results showed that the studied orchids species have cardiac glycosides, phenolic compounds, flavonoids, coumarins, flavanones, proteins and quinones as secondary metabolites. Those phytochemicals are used in the defence mechanisms by the plants, and they are detected in most medicinal plants [19]. In recent years, the roles of those secondary metabolites are increasing due to human nutrition [31]. Flavanones, particularly important for human cancer researches. It is known that this secondary metabolite is presence in the roots of the orchid *Spiranthes australis* [32] Growth inhibition effect of flavanones on the human cancer cells was studied by researchers [33].

Quercetin and ferulic acid are known as antioxidants found in salep orchids [34]. Consumption of natural antioxidants, such as quercetin and ferulic acid, protects sperm cells against oxidative stress and improves fertility [35]. We tested the extract doses ranging from 20-100 µg. We observed the best $DPPH^{\bullet}$ scavenging activity was in chloroform extract of *O. speculum* with 35.12 ± 0.58% at 100 µg. This supports the traditional effect of the salep on fertility. In further antimicrobial activity analyses, *A. morio n*-hexane extract and *A. pyramidalis* methanol extract and water extract were the most effective plant extracts and showed bacteriostatic activity against the clinical pathogenic bacteria *P. vulgaris* and the others, while *N. tridentata* extracts have the least. Isoflavones, which have high antimicrobial activity, are dissolved in *n*-hexane, methanol and water solvents [36]. The reason for higher antimicrobial activity of *A. morio* and *A. pyramidalis* could depend on high isoflavone level. In the study of [25], chloroform extract of the orchid *Cymbidium aloifolium* (L.) SW., which is not being used for producing salep, is the most effective on test pathogens, while *n*-hexane extracts are the least effective. In another study by [37], the petroleum ether, chloroform, ethyl acetate and methanol extracts of the medicinal orchid *Malaxis rheedei* SW. have antimicrobial activity against selected microorganisms. Orchids are well known for their symbiotic relationships with mycorrhizal fungi [38]. The balance between orchids and the mycorrhizal fungi is dependent on this two-sided battle. If fungi win, the endosperm-poor seeds die. And if the seeds win, the fungi die and germination never starts. Thus, the strong but sufficient antifungal activity of the orchids, which are full heterotrophic during their germination stage, remains throughout its life time. In our results, *N. tridentata* extracts (except *n*-hexane extract) and *O. speculum* chloroform extract showed strong antifungal inhibition against *C. albicans*.

5. CONCLUSIONS

This study reports the phytochemical analysis, antioxidant and antimicrobial activities of various extracts of *A. morio*, *A. pyramidalis*, *N. tridentata*, *O. mammosa*, *O. lutea* and *O. speculum* orchid plants for the first time, and it has been shown that the plants have bioactive components. Consuming salep obtained from these orchids can provide antioxidants and antimicrobial compounds for human health.

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