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Polyphenols from Plants: Phytochemical Characterization, Antioxidant Capacity, and Antimicrobial Activity of Some Plants from Different Sites of Greece

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Abstract: Polyphenols are present in many plants and herbs, and the scientific community and consumers are aware of their health-promoting effects. Plants of Greek origin were studied for their polyphenol content and their antioxidant and antimicrobial activities. Gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography coupled to a diode array detector (HPLC–DAD) were used for the identification and characterization of plant polyphenols. For GC–MS, a silylation procedure was employed. Ferulic acid, quercetin, and catechin were the most abundant polyphenols. The Rancimat test, FRAP (Ferric-reducing Antioxidant power) assay, and DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay were used to study the antioxidant capacity, which was proven for all studied plants. The antimicrobial activity was studied against specific pathogenic microorganisms. *Pelargonium purpureum* and *Sideritis scardica* plant extracts inhibited most microorganisms such as *L. monocytogenes* and *E. coli*. Extracts of studied plants showed both antioxidant and antimicrobial activities; hence, they can be considered to be used by the food industry.

Keywords: Greek plants; bioactive compounds; silylation; antioxidants; Rancimat test; antimicrobial activity; antioxidant activity

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1. Introduction

Antioxidants are commonly used in foods to inhibit lipid oxidation since rancid odors and flavors decrease nutritional quality [1]. Plant antioxidants can be effective against lipid oxidation, and both herbs and spices are being used for this reason [2].

In this study, Greek plants of the *Lamiaceae* family were studied via HPLC–DAD and GC–MS after silylation. DPPH and FRAP assays, Rancimat test, and total phenolic content (TPC) were employed to study antioxidant capacity.

GC, HPLC, and capillary electrophoresis (CE) are common methods to determine polyphenols [3–5]. The last two do not require derivatization, but for GC, this is obligatory. Even though these techniques are popular in the scientific community, detectors such as UltraViolet-Visile (UV–Vis) and diode array detectors, cannot be used for compound characterization [6,7]. Hence, GC coupled with mass spectrometry (MS) can be used for providing more accurate data. It may provide the molecular masses and fragmentation patterns of compounds present in samples. Trimethylsilyl (TMS) derivatives of polyphenols have been prepared for GC–MS analysis [4].

Chromatographic methods and MS can be combined so as to characterize specific polyphenols and bioactive compounds in general. The aim of this study was to determine the phenolic compounds present in the extracts of Greek aromatic plants using HPLC–DAD and GC–MS. Different solvents were used and compared. The antioxidant and antimicrobial capacities/activities of these plant extracts are presented as well using different assays.

Greek producers and consumers, as well as the food and cosmetic industry, will gain knowledge on the content and activity of these compounds that are used in Greek cuisine, and they can be utilized by these industries to obtain bioactive compounds or replace the currently used chemical preservatives.

2. Materials and Methods

2.1. Standards

All standards used were purchased from Sigma (Steinheim, Germany). Quantification was performed by the use of standard compound calibration curves.

2.2. Solvents and Reagents

All solvents were purchased from Sigma (Steinheim, Germany). Only Folin–Ciocalteu reagent and silylation reagents were purchased from Merck (Darmstadt, Germany), Water was supplied by a Milli-Q water purifier system from Millipore (Bedford, MA, USA).

2.3. Plant Material

Dried samples (air-dried below 40 °C) were obtained commercially from Athens, Greece. *Filipendula ulmaria*, *Rosmarinus officinalis*, and *Geranium purpureum* were collected from Pindus Mountain in northern Greece, while *Salvia Officinalis* was collected from mount Menalon, Arcadia, Greece, and *Sideritis scardica* from Mount Olympus, central Greece. Leaves of all samples were analyzed within 3 months of collection.

2.4. Sample Preparation and Derivatization

The protocol used for extraction has been previously reported [8]. Aqueous methanol 62.5% v/v was used for extraction.Butylated Hydroxy Toluene (BHT)was used during extraction to prevent the decomposition of plant polyphenols [9].

2.5. HPLC Analysis

HPLC–DAD analysis was employed according to a previously reported protocol [10]. In brief, a JASCO (PU-980, JASCO International, Co., Ltd., Tokyo, Japan) HPLC system was used with a diode array detector (DAD, MD 910, JASCO International, Co., Ltd., Tokyo Japan) and a Waters Spherisorb® column (5μm ODS2 4.6 × 250mm) at 25 °C. The flow rate was 0.5 mL/min, and the injection volume was 20 μL. Retention time and spectral matching were combined to detect polyphenols. Phenolic acids were monitored at 280 nm and flavones and flavonols at 320 and 370 nm, respectively.

2.6. GC–MS Analysis

Polyphenols from plant extracts were analyzed after silylation using the method previously described by authors [8] by the use of TMCS (100 μL) and BSTFA (200 μL). Wiley and NIST libraries (*NIST* 20 (2020)) were used to characterize phenolic compounds.

2.7. Rancimat Test

The Rancimat test was performed according to the previously reported method by the authors [1,9]. The protection factor (PF) was measured based on the following formula: $PF = IP_{extract}/IP_{control}$.

2.8. Determination of Total Phenolic Content of Plant Extracts

TPCs were determined via the Folin–Ciocalteu assay and are expressed in milligrams of gallic acid per gram of dry sample [11].

2.9. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

This procedure was performed based on previous relevant works [12–14]. IC_{50} values of extracts were calculated and are expressed in μg/mL. Ascorbic acid and BHT were used as standards to compare our samples.

2.10. Ferric-Reducing Antioxidant Power (FRAP)

The FRAP protocol was carried out according to previous relevant studies [15–17]. Changes in absorptions were measured at 0, 4, and 30 min as ΔA values.

2.11. Antimicrobial Assay

Plant extracts were tested against specific pathogenic microorganisms presented in Table 5 according to a previously reported method in triplicate, and inhibition zones (IZs) were measured by the use of a SONY camera (x-wave HAD SSC-DC50AP, SONY Professional Solutions, Europe, Surrey, UK) [11].

2.12. Statistical Analysis

Statistical analysis was conducted using Minitab 13.1 for Windows, MINITAB LTD, Coventry, UK. Mean values of samples were compared using MANOVA (MICROSOFT, Excel 2019). Tukey's test for mean values significant difference was employed.

3. Results and Discussion

3.1. Antioxidant Capacity

PF values from the Rancimat test and the TPC content are presented in Table 1. The highest concentration was detected in *Salvia officinalis* and the lowest in *Sideritis scardica*. The results were comparable to those reported in a similar study [12].

Table 1. TPC and antioxidant activity.

Species	Part Examined	Drying Method [a]	Total Phenolics [b] (mg Gallic Acid/ g ds)	PF [c] (Ground Sample)	PF (Methanol Extracts)	IC_{50} [d] (mg/L)
Filipendula ulmaria	Flower	Air	77.4 ± 3.71	1.3	1.2	47.0 ± 2.1
Salvia officinalis	Herb	Air	234.7 ± 12.9	1.5	1.4	23.4 ± 1.4
Rosmarinus officinalis	Herb	F/v	190.2 ± 6.3	1	0.8	34.9 ± 2.2
Sideritis scardica	Leaves	F/v	75.3 ± 2.1	1.8	1.7	49.6 ± 0.4
Geranium purpureum	Leaves	F/v	120.5 ± 3.7	3.1	2.9	41.3 ± 2.1

[a] Air = air drying; F/v = freeze–vacuum, i.e., lyophilization. [b] Mean of duplicate assays; ds = dry sample; [c] PF = protection factor from Rancimat test; [d] IC_{50} = inhibitory concentration 50.

The Rancimat test results revealed that plant extracts may protect against lipid oxidation, as sunflower oil was used as an indicator oil in this study. Antioxidants can be either "preventive" antioxidants or "chain-breaking" antioxidants [18]. With the addition of ground plant samples to sunflower oil, PFs were slightly higher than after the addition of methanol extracts. Gallic acid had PF = 4.5, which was the highest value, followed by (+)-catechin and (−)-epicatechin, with values of 1.8 and 2.5, respectively. Flavonoids had similar PF values, ranging from 1 to 1.2. The determined PF values were in line with TPC values.

3.2. HPLC Analysis

A similar technique to the one applied in this study has been published for polyphenols [19,20]. The concentration of phenolic acids detected in the analyzed samples after acid hydrolysis is presented in Table 2. Table 3 presents the results for plant flavonoids in mg/100 g of dry sample (ds).

Gallic acid was determined in all plant samples except *Filipendula ulmaria*. Gentisic acid and Syringic acid were only detected in *Geranium purpureum.* Hydroxytyrosol was also detected, reported mainly in *Geranium purpureum* (11.2 mg/100 g dry sample) even though it is more relevant to olives [21,22]. Ferulic (in *Filipendula ulmaria)* and caffeic acids were the phenolic acids with higher concentrations. Catechin, rutin, and quercetin were the main flavonoids detected. Apigenin was present only in *Salvia officinalis* (Table 3). Similar to herbs, plants contain comparable amounts of polyphenols [22]. Quercetin, luteolin, and apigenin have been reported in Malaysian tropical plants [23]. Acid hydrolysis undoubtedly

affected the final concentration of polyphenols and phenolic acids determined in this study since most of the phenolic compounds were found in glycosidic forms in plant matrices. Some other researchers employed basic hydrolysis which can be also used for polyphenol release from plant matrices [24]. Therefore, phenolic compounds are present mainly as esters and glycosides rather than in free form [25]. This is why hydrolysis was performed prior to HPLC–DAD. There are scarce data in the literature concerning the polyphenol and phenolic acid contents of the specific plants examined; however, these compounds are very important in Greek cuisine and traditional applications, and therefore, their analysis is considered important. Data presented in Tables 2 and 3 depend on the analytical method applied and are indicative.

Table 2. Phenolic acid concentrations of plant extracts studied.

	mg/ 100g ds [a]							
Plant	**GLA**	**GA**	**CA**	**pCA**	**VA**	**SA**	**FA**	**pHBA**
Filipendula ulmaria	ND	ND	6.5 ± 0.02	4.3 ± 0.02	4.8 ± 0.02	ND	13.2 ± 0.04	ND
Salvia officinalis	3.4 ± 0.01	ND	ND	ND	ND	ND	6.7 ± 0.02	ND
Rosmarinus officinalis	1.2 ± 0.02	ND	4.9 ± 0.01	ND	ND	ND	4.8 ± 0.01	ND
Sideritis scardica	3.7 ± 0.01	ND	5.3 ± 0.03	ND	ND	ND	4.2 ± 0.02	ND
Geranium purpureum	14 ± 0.02	3.2 ± 0.03	20 ± 0.03	4.1 ± 0.02	2 ± 0.02	1.1 ± 0.02	ND	5.4 ± 0.02

[a] Each value is the mean (mg/100g dry sample) of two replications; ±standard deviation; ND = not detected. GLA, GA, CA, pCA, VA, SA, FA, and pHBA stand for gallic acid, gentisic acid, caffeic acid, p-coumaric acid, vanillic acid, syringic acid, ferulic acid, and p-hydroxybenzoic acid, respectively.

Table 3. Flavonoid concentration of plant extracts studied.

	Content, mg/100g Dry Sample [a]								
Plant	**Quercetin**	**Apigenin**	**Luteolin**	**Naringenin**	**Eriodictyol**	**Rutin**	**(+)-Catechin Hydrated**	**(−)-Epicatechin**	**Hydroxytyrosol**
Filipendula ulmaria	1.5 ± 0.01	ND	ND	ND	ND	1.3 ± 0.01	ND	ND	ND
Salvia officinalis	3.6 ± 0.01	4 ± 0.01	ND	1.7 ± 0.02	ND	2.6 ± 0.01	ND	ND	ND
Rosmarinus officinalis	ND	ND	3.1 ± 0.02	ND	0.3 ± 0.02	ND	ND	ND	ND
Sideritis scardica	3.6 ± 0.02	ND	ND	ND	ND	1.9 ± 0.01	ND	4.5 ± 0.04	ND
Geranium purpureum	11.2 ± 0.02	ND	ND	ND	ND	4.5 ± 0.01	1.5 ± 0.01	5.6 ± 0.02	11.2 ± 0.04

[a] Each value is the mean (mg/100g dry sample) of two replications; ±STD deviation; ND = not detected.

3.3. GC–MS Analysis

The derivatization reaction is a nucleophilic substitution reaction [4]. Trimethylchlorosilane (TMCS) was used to increase the derivatization process.

Usually, 1% TMCS in BSTFA can achieve the desired derivatization [26]. BSTFA was also used in similar cases such as wines and white juices [27].

A similar technique has been applied in *Ginkgo biloba* L. extracts [28]. Molecular weights (MW) and important identified ions of the silylated polyphenols are presented in Table 4, using the Wiley and NIST libraries. The method applied in this study (GC–MS) is considered a semi-quantitative method since some polyphenols such as luteolin, naringenin apigenin and eriodictyol had very poor derivatization sensitivity even at high concentrations such as 30 mg/L. However, this analytical technique helped us to separate and characterize most of the studied compounds, and the resolution was excellent. It is worth noting that special care is needed when performing analyses such as employing anhydrous conditions and deactivation of glassware with 5% dimethyldichlorosilane (DMDCS) in toluene. Another advantage is that cinnamic acid, protocatechuic acid, and p-hydroxyphenylacetic acid that are not determined by using HPLC–DAD were detected with this method as TMS derivatives in the libraries used (NIST and Wiley).

3.4. DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Assay

IC_{50} values of hop extracts showed similar values to BHT and ascorbic acid (Table 1). IC_{50} values of all plant extracts (extracted with methanol, water, and dichloromethane)

were determined using curves plotted similarly to the ones of *Salvia officinalis* presented in Figure 1.

Higher antioxidant capacity was found in methanolic extracts than in other solvents. The lower the value of EC_{50}, the higher the antioxidant capacity. Concerning the scavenging of this commercially available free radical, methanol was the solvent that gave better results than the other used solvents.

Table 4. Molecular weights (MWs) and identified ions of compounds detected with GC–MS.

Compound *	MW (Silylated Compounds)	Identified Ions (*m*/*z*)
p-HBA	282	267 (100%), 193, 223, 282
VA	312	149 (100%), 312, 223, 165
GA	370	355 (100%), 281, 147, 223, 267, 370
GLA	458	281 (100%), 458, 179, 147
pCA	308	219 (100%), 293, 308, 249
FA	338	338 (100%), 308, 323, 249, 293, 219, 279
CA	396	219 (100%), 396, 381, 191
Quercetin	647	575 (100%), 647, 487
Catechin	650	368 (100%), 355, 650, 267, 383, 179, 297
Hydroxytyrosol	370	267 (100%), 193, 179, 370
SA	342	327 (100%), 312, 297, 342
Epicatechin	650	368 (100%), 355, 267, 147, 649
p-Hydroxyphenylacetic acid	296	179 (100%), 164, 149, 296
Protocatechuic acid	370	193 (100%), 223, 370, 267
Cinnamic acid	220	131 (100%), 205, 103 161, 220

* Identified as trimethylsilyl (TMS) derivatives. GLA, GA, CA, pCA, VA, SA, FA, and pHBA stand for gallic acid, gentisic acid, caffeic acid, p-coumaric acid, vanillic acid, syringic acid, ferulic acid, and p-hydroxybenzoic acid, respectively.

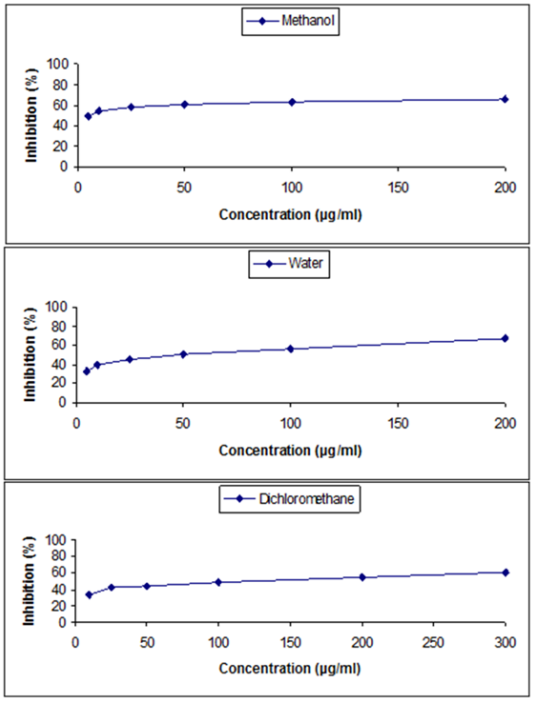

Figure 1. %DPPH radical scavenging expressed as inhibition (%) vs. concentration (µg/mL) curves for IC_{50} values determination of *Salvia officinalis* extract.

3.5. Ferric-Reducing Antioxidant Power (FRAP)

Some flavones and flavanones that have high FRAP values have shown prooxidant activities [29]. In this study, the absorbance of the plant extracts after extraction with four different solvents was measured at times 0 and after 4 and 30 min. The change in

absorbance ($\Delta A = A_{4min} - A_{0min}$ and $\Delta A = A_{30min} - A_{0min}$) was calculated. Figure 2 presents the change in absorbance from 0 to 30 minutes for all solvent extracts, as well as the effect of the solvent on this change (ΔA) for the two-time measurements (4 and 30 min).

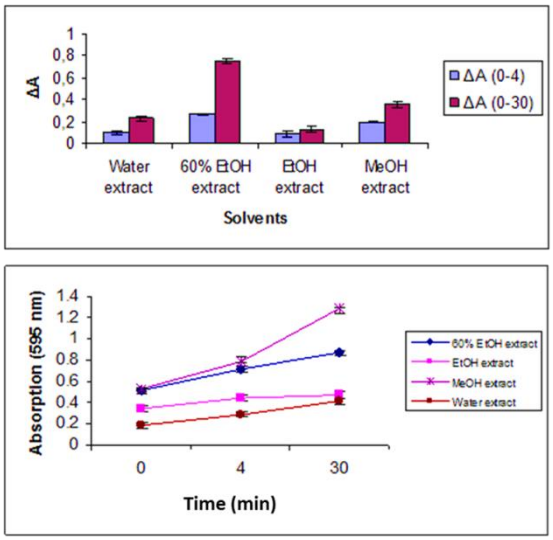

Figure 2. Change in absorbance (presented as $\Delta A(0–4) = A_{4min} - A_{0min}$ and $\Delta A(0–30) = A_{30min} - A_{0min}$) and absorption readings taken at 0, 4, and 30 min for *Salvia officinalis* extract at 595 nm for 50% EtOH, EtOH, MeOH, and water extract.

Only the 60% ethanol hop extract had significantly higher ($p < 0.05$) changes than the other extracts. In addition, the 60% ethanol and methanol extracts significantly changed the reducing capacity of hop polyphenols based upon ferric ion. The antioxidant activity can vary and depends on the analysis time [30]. In this study, an analysis range of 0 to 30 min was considered adequate to check and evaluate the reducing power of plant extracts. FRAP is considered a routine analysis assay, as it is cheap and requires no expensive instrumentation, similar to DPPH, and researchers can obtain fast results concerning the antioxidant activity of samples.

3.6. Antimicrobial Activity

The disk diffusion method was used for measuring antimicrobial activity. Similar techniques and pathogens have been reported [31,32].

The antimicrobial activity of plant extracts is presented in Table 5. *Geranium purpureum*, *Sideritis scardica,* and *Rosmarinus officinalis* had significant effects on microorganisms. *Listeria monocytogenes* ScottA was the most sensitive microorganism to the plant extracts examined in this study. *Salmonella enteritidis* PT4 was not affected by the extracts. Gram (+) bacteria were more sensitive to the studied plant extracts than Gram (−) bacteria such as *Enterobacteriaceae* (*Escherichia coli* 0157:H7 NCTC12900 and *Salmonella enteritidis* PT4).

Table 5. Plant extract's antimicrobial activity (sample amount 5μL; n = 3).

Plant Extracts	*Escherichia coli* 0157:H7 NCTC12900	*Salmonella enteritidis* PT4	*Staphylococcus aureus* ATCC 6538	*Listeria monocytogenes* ScottA	*Bacillus cereus* FSS134	*Pseudomonas putida* AMF178
Filipendula ulmaria	- [a]	-	~ [b]	++ [d]	~	+ [c]
Salvia officinalis	~	-	-	++	~	~
Rosmarinus officinalis	-	-	-	+	-	+
Sideritis scardica	+	-	-	++	-	+
Geranium purpureum	++	-	+	++	+	++

[a] - No antimicrobial capacity, IZ of sample < IZ of solvent (62.5% aqueous methanol); [b] ~ Slight antimicrobial capacity, IZ of sample 1–3 mm > IZ of solvent; [c] + Moderate antimicrobial capacity, IZ of sample 3–4 mm > IZ of solvent; [d] ++ Clear antimicrobial capacity, IZ of sample 4–10 mm > IZ of solvent.

4. Conclusions

In this study, the antioxidant and antimicrobial activity of several Greek plant extracts was analyzed by the application of the Rancimat test and routine assays such as FRAP and DPPH. Concerning the antimicrobial activity, Gram (+) bacteria were more sensitive to the plant extracts than Gram (−) bacteria. The most potent antimicrobial activity was shown by *Pelargonium purpureum* and *Sideritis scardica* plant extracts. The most abundant polyphenols in the plant extracts were found to be catechin, ferulic acid, and quercetin, as measured by HPLC. A semiquantitative technique, namely GC–MS, was used to characterize phenolic acids and polyphenols and investigate whether this technique can be used as an alternative to HPLC. It was proved that some compounds were detected using this technique and not through HPLC. However, there are limitations such as the conditions that need to be applied and that some phenolic compounds could not be silylated and detected even at high concentrations. The obtained results indicated that the analyzed plant extracts may become important, cheap, and noticeable sources of bioactive compounds, such as polyphenols and phenolic acids, and at the same time, have antioxidant and antimicrobial activities; therefore, they have potential use in the food, nutraceutical, and pharmaceutical industries.

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