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Article

Identification and Determination of Phenolics in *Lamiaceae* Species by UPLC-DAD-ESI-MS/MS

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Abstract

This study reports the phenolic profile screening of aromatic *Lamiaceae* species such as marjoram (*Origanum majorana* L.), lavender (*Lavandula officinalis*) and pennyroyal (*Mentha pulegium* L.) using a novel and validated ultra performance liquid chromatography method coupled with DAD diode array detector and tandem mass spectrometry (MS/MS) in negative mode of electrospray ionization. Identification and quantification of phenolics in these plant extracts has been realized within 12 min. This method showed good precision (percentage relative standard deviation; RSD% 0.54–2.72 for intra-day, 1.71–4.64 for inter-day), reproducibility (percentage recovery, REC% 92.0–109.0) and linearity (r = 0.9988–0.9999). Limits of detection ranged from 0.02 to 18.2 ng/mL. The extraction of plants was performed using microwave-assisted extraction technique and 60% (v/v) aqueous methanol solvent medium was selected as suitable solvent because of maximum extraction efficiency. Total antioxidant capacity, total phenolic content and free radical scavenging activity of these plant extracts were tested and the results correlated well among each other. According to the Folin assay, phenolic contents of *Origanum majorana* L., *Mentha pulegium* L. and *Lavandula officinalis* were calculated as 119 ± 3.4, 85.1 ± 2.8 and 57.8 ± 2.1 mg GAE/g dry matter, respectively.

Introduction

Phenolic compounds (i.e., flavonoids, phenolic acids, tannins, lignans) constitute a large group of secondary plant metabolites. These compounds have medicinal and beneficial properties against many chronic diseases, such as cancer, aging, diabetes and degenerative diseases (1). They can be utilized for preventing oxidative damage by scavenging free radicals, chelating catalytic metal ions, quenching singlet oxygen and reducing reactive species (2). Hence, there is an increasing attention to polyphenols due to their considerable impacts on human health.

Lamiaceae is one of the large plant families used as a framework to evaluate the occurrence of some typical secondary metabolites (3). Extracts of aromatic *Lamiaceae* species such as *Origanum majorana* L., *Mentha pulegium* L. and *Lavandula officinalis* contain natural polyphenolic antioxidants and show high antioxidant capacity (4–8). Pennyroyal, marjoram and lavender extracts have been known as important dietary sources in addition to their use for medicinal purposes. Pennyroyal is an aromatic herb that has been traditionally used in medicine (digestive, amenorrhea, colds, liver disorders, skin diseases, gout), aromatherapy, cosmetics and gastronomy (culinary herb) (9, 10). Marjoram has been traditionally used for the treatment of gastrointestinal disturbances, cough and bronchial diseases, in mouthwashes for oral hygiene and to relieve symptoms of the common cold, such as nasal congestion (11). Lavender has generally been considered as a sedative, antidepressive, diuretic, anticonvulsant, antibacterial, antispasmodic, antiflatulent, antiemetic and tonic (12).

According to previous studies, Zugic *et al.* (7) examined that apigenin, isoquercitrin and luteolin-7-O-glucoside were identified in *Mentha pulegium* L. The presence of rosmarinic acid, caffeic acid, hispidulin and

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apigenin was reported in *Origanum majorana* L. (13). Proestos *et al.* (4) found certain phenolic acids (ferulic, caffeic, gentisic and *p*-hydroxybenzoic acid) and flavonoids (apigenin and catechin) in the methanolic extracts of *Origanum majorana* L. herbs. In another study, reversed phase - high performance liquid chromatography analysis of the methanolic extract of *Origanum majorana* L. dried aerial parts was carried out with respect to growth stages. *Trans*-2-hydroxycinnamic, rosmarinic, vanillic, chlorogenic, gallic and cinnamic acids were identified as main phenolic acids whereas the major flavonoids were amentoflavone, apigenin, quercetin, luteolin, coumarin and rutin (5). Taamalli *et al.* performed the characterization of phytochemicals present in the methanolic extracts of *Origanum majorana* L. and *Mentha pulegium* L. by ultra performance liquid chromatography (UPLC) coupled electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS) detection. The presence of syringic, *p*-coumaric, rosmarinic acid and hesperidin was reported in both extracts (14). According to the study of Proestos *et al.* (15), gallic acid, *p*-hydroxybenzoic acid, catechin, vanillic acid, caffeic acid, ferulic acid and naringenin were detected in lavender. Areias *et al.* (6) reported that *o*-coumaric acid, coumarin, rosmarinic acid, herniarin, luteolin and apigenin were detected in ethanolic extracts of *Lavandula officinalis*.

UPLC-MS/MS is an advanced technique facilitating high sensitivity, resolution, reproducibility and speed. Several researchers have developed novel analytical methods for screening the phenolic profile of different herbal plant and/or fruit extracts using UPLC with ESI tandem mass spectrometry (MS/MS) (16–21), MS (22) and UV/Vis (23) or PDA detection (24, 25). Microwave extraction is an efficient technique which plays an important role in separation of bioactive compounds from plant matrices. Optimization of process parameters (microwave power, temperature, time, solvent type) can significantly improve extraction efficiencies (26). In recent decades, certain procedures with reduced extraction time and enhanced extraction yield were developed for polyphenols recovery from different plants (27, 28).

This study reports original information, as the phenolic profile of microwave-assisted extracts of three herbal plants were reported in detail using a sensitive and reliable MS-coupled UPLC-chromatographic method for the first time. Compared with the HPLC method, the novelty of the method was mainly in its application to a wide group of polyphenolic compounds assayed in a shorter time. Total antioxidant capacity (TAC), total phenolic content (TPC) and free radical scavenging activities of the plant extracts were also evaluated.

Experimental

Chemicals and reagents

Chemical substances of analytical reagent grade were supplied from the sources given below: apigenin (≥95% purity), naringenin (≥95% purity), morin (≥85% purity), quercetin (≥98% purity), caffeic acid (≥98% purity), rutin (≥94% purity), kaempferol (≥90% purity), gallic acid (≥98% purity), neocuproine (Nc), methanol (≥99,9% purity), formic acid (98–100% purity), DPPH (2,2-diphenyl-1-picrylhydrazyl) and Folin–Ciocalteau reagent were purchased from Sigma Co. (St. Louis, MO); *p*-coumaric acid (≥98% purity), vanillic acid (≥97% purity), ferulic acid (≥99% purity), rosmarinic acid (≥96% purity) were purchased from Aldrich Chemicals Co. (Steinheim, Germany); protocatechuic acid (≥97% purity), sinapic acid (≥98% purity), fisetin (≥99% purity) and catechin (≥98% purity) were purchased from Fluka (Buchs, Switzerland). Copper(II) chloride, ethanol (≥99.8% purity), ammonium acetate, syringic acid (≥96% purity), sodium potassium tartarate (99% purity), copper(II) sulfate (≥98% purity) and sodium carbonate (≥99% purity) were purchased from Merck (Darmstadt, Germany). The purity assay for each standard substance was performed by HPLC except for ferulic acid, chlorogenic acid, gallic acid and sinapic acid, which were in turn made by alkalimetric titration. Herbal plants (*Mentha pulegium* L., *Origanum majorana* L., *Lavandula officinalis*) were purchased from a local herbalist (Istanbul, Turkey). The country of origin of the plants was Turkey. These plants were cultivated in various parts of Anatolia. Leaves and aerial parts of marjoram (*Origanum majorana* L.) from the town of Alanya (city of Antalya) were collected between June and July, 2013. Flowers of lavender (*Lavandula officinalis*) from Keçiborlu town (city of Isparta) and leaves of pennyroyal (*Mentha pulegium* L.) from Karacadağ town (city of Şanlıurfa) were harvested before flowering stage between April and June, 2013. The taxonomic identification of these plants was approved by academic staff from the Division of Botany of Istanbul University. The samples were air dried at ambient temperature (~25°C) and milled.

Instruments

The spectra and absorption measurements were recorded in matched quartz cuvettes using a Perkin Elmer Lambda 35 UV-Vis spectrophotometer (Shelton, CT, USA). UPLC analysis was performed using a Waters Acquity (Milford, MA, USA) equipped with a binary solvent manager, sample manager, column thermostat and double quadrupole analyzer. The ETHOS-One (Milestone, Shelton, CT, USA) closed vessel oven system equipped with 12 teflon (PTFE) vessels and an automatic fiber optic temperature control system was used for microwave-assisted extractions (MAE).

Preparation of solutions

The stock solutions of polyphenols at 10 mM concentration were prepared in methanol and kept at −20°C prior to analysis. Ten millimolar $CuCl_2$ solution and 1 M pH = 7 ammonium acetate solution were prepared in distilled water (Millipore Simpak1 Synergy 185, USA); 7.5 mM neocuproine solution was prepared in absolute ethanol. About 50 mL of Lowry A, composed of 2% (w/v) aqueous Na_2CO_3 in 0.1 M NaOH, was freshly mixed with 1 mL of Lowry B, composed of 0.5% (w/v) $CuSO_4$ aqueous solution in 1% (w/v) $NaKC_4H_4O_6$ solution, to prepare Lowry C solution. Lowry C was used in admixture with Folin–Ciocalteau reagent (prepared by diluting the commercial reagent at 1:3 volume ratio with H_2O) in the Folin–Ciocalteau assay. The DPPH radical solution (1 mM) was prepared in ethanol and diluted with ethanol by adjusting the absorbance of the DPPH radical solution to 0.750–0.900 AU at 525 nm.

MAE of plant extract

Extraction procedure was performed with various solvents (methanol, ethanol, acetone) at different ratios (80% and 60%; v/v) at 80°C and 500 W. The herbal plants were extracted using the method previously reported (29) with slight modifications: Dried plant sample (1.0 g) in 15 mL aqueous methanol solution was placed in the inner vessel. A magnetic stirring rod was added into each vessel. The vessels were placed symmetrically in the microwave field. The suspensions were irradiated with microwaves as follows: 3 min for heating to the desired temperature (80°C), 3 min for balancing at

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80°C and 5 min for cooling. This protocol was repeated using all solvent media mentioned above. Plant extracts were filtered through a 0.45 µm micro-filter (Whatman, UK) and kept at +4°C until use. All extractions were performed in duplicate.

UPLC-DAD-ESI-MS/MS analysis

An Acquity UPLC system coupled to diode array detector (DAD) and Xevo TQD (double quadrupole analyzer) mass spectrometer (Waters) equipped with Z-spray ESI source operating in negative mode were employed for direct injection analyses. The analyses were performed using Acquity BEH C18 analytical column (100 × 2.1 mm, 1.7 µm) (Milford, MA, USA). To analyze phenolic compounds, the mobile phase consisted of two solvents, i.e., 0.1% of formic acid in bidistilled water (A) and 0.1% of formic acid in methanol (B). The polyphenolic antioxidants were analyzed using gradient elution: (Flow rate = 0.45 mL/min; column temperature: 30°C): 0. min 90% A–10% B; 2. min 85% A–15% B (slope 1.0); 3. min 80% A–20% B (slope 1.0); 4. min 70% A–30% B (slope 1.0); 6. min 60% A–40% B (slope 1.0); 8–10. min isocratic elution (50% A–50% B (slope 1.0)); 12. min 90% A–10% B (slope 1.0). Detection wavelength was set from 190 to 400 nm. All injected solutions were stored at 4°C in the auto-sampler. Injection volume was set to 10 µL in partial loop with needle overfill (PLINO) mode. The ionization working conditions were set for Xevo TQD mass spectrometer as follows: capillary voltage, 3 kV; collision energy, 20 V; cone gas flow rate, 50 L/h; desolvation temperature, 400°C; desolvation gas flow rate, 650 L/h. Cone voltage and collision energy were optimized for each compound by using IntelliStart™ software (Waters, Milford, MA, USA). Data acquisition was accomplished using MassLynx™ v 4.1 software (Waters, Milford, MA, USA). Polyphenols were identified using mass-to-charge (m/z) transitions of precursor and product ions in multiple reaction monitoring (MRM) mode. Using the above working mode, these polyphenolic compounds were identified by matching retention times, UV-vis and mass spectral data with those of standards concerned. The calibration curves were established for the polyphenols of interest as peak area versus concentration.

Validation of chromatographic method

The proposed chromatographic method was validated by applying method suitability tests such as linearity, LOD, LOQ, precision and accuracy. Linear concentration range was studied using mixed standard solutions ranging from 1.5 to 6,100 ng/mL. Calibration curves for concentration (7 different concentrations) versus response ratios were plotted for each analyte and slope (a), intercept (b) correlation coefficient (r) were evaluated. The limit of detection (LOD) and limit of quantification (LOQ) were calculated at the critical signal-to-noise (S/N) ratios of 3 and 10, respectively (Table II). The precision of the method was evaluated by spiking herbal plant extract with standards at final concentrations of 5 µM. For the intra-day variations, herbal plant extract spiked with mixed standard solution of 18 analytes was injected in six replicates in 1 day; for the inter-day variations, the spiked sample was injected in six replicates over 3 consecutive days. Variations were expressed as the percentage relative standard deviation (RSD%) of the replicates. The test for recovery was carried out with herbal plant extracts spiked with mixed standard solutions at 10 µM to examine the accuracy of the proposed method, where three replicates were studied in each case.

CUPRAC TAC assay

The CUPRAC method (30) is based on the reduction of a cupric neocuproine complex (Cu(II)-Nc) by antioxidants to the colored cuprous chelate (Cu(I)-Nc). CUPRAC reagent comprised of 1 mL each of copper(II) chloride, neocuproine and ammonium acetate buffer solution were prepared. About 25 or 50 µL of the dilute plant extract (diluted with 60% MeOH at a volume ratio of 1:10) and the necessary amount of H_2O were added to the CUPRAC reagent to make the final volume: 4.1 mL. The absorbance at 450 nm (A_{450}) was recorded against a reagent blank after 30 min. The method was applied thrice to the plant extracts. Trolox was used as the standard control. The trolox equivalent TAC was calculated with the use of the molar absorptivity of trolox (ε_{TR} being 1.58×10^4 $Lmol^{-1}cm^{-1}$ under the specified conditions; optical cuvette thickness = 1 cm). TAC of plant sample can be estimated by using the calculation given below:

TAC (mmol TR/g) = $(A_{450}/\varepsilon_{TR})$ (total volume/taken extract volume) (dilution factor) (extract volume/g dry weight of plant).

Determination of TPC

TPC of the methanolic extract was determined using the Folin–Ciocalteau method as described by Singleton *et al.* (31). To x mL of the dilute plant extract (diluted with 60% MeOH at a volume ratio of 1:10) were added an aliquot of 2.5 mL of Lowry C solution and $(1-x)$ mL H_2O, and the mixture was let to stand for 10 min. At the end of this period, 0.25 mL of Folin–Ciocalteau reagent was added, and 30 min was allowed for stabilization of the blue color formed. The absorbance against a reagent blank was measured at 750 nm. The result was converted to gallic acid equivalent (mg GAE/g-sample) unit based on the calibration curve obtained using gallic acid standards. The molar absorptivity of gallic acid in the Folin–Ciocalteau method was $\varepsilon_{GA} = 5.73 \times 10^3$ $Lmol^{-1}cm^{-1}$ (32). The assays were carried out in triplicate and the results expressed as (mean ± standard deviation (SD)).

DPPH free radical scavenging activity

The scavenging activity of the methanolic extract on DPPH radicals was measured according to the method of Sánchez-Moreno *et al.* (33) with minor modifications. One mL of ethanolic DPPH radical solution (0.5 mM) was added to the mixture solution of x mL extract and $(4-x)$ mL ethanol. The mixture was incubated for 30 min at room temperature. At the end of this period, the absorbance was recorded at 525 nm. The free radical scavenging activity was calculated as the percentage of DPPH decolorization using the equation: % = $((A_{DPPH} - A_{sample})/A_{DPPH}) \times 100$, where A_{DPPH} was the initial absorbance of DPPH radical solution without sample, and A_{sample} was the absorbance of the solution after addition of the sample extract at a particular level. The assays were carried out in triplicate and the results expressed as (mean value ± SD). The related concentration of plant extract to decrease the absorbance of DPPH radical solution by 50% (IC_{50}) was calculated graphically.

Results

Optimization of chromatographic analysis

Screening of phenolic composition of pennyroyal (*Mentha pulegium* L.), marjoram (*Origanum majorana* L.) and lavender (*Lavandula officinalis*) extracts utilizing a novel UPLC-based analytical method equipped with DAD and MS/MS detectors was performed. Eighteen

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polyphenols were seperated by UPLC gradient elution program in the presence of 0.1% of formic acid in bidistilled water and 0.1% of formic acid in methanol (Figure 1). MS data were acquired in negative ionization mode in which deprotonated ions $(M–H)^-$ were analyzed. MS/MS parameters such as MRM transitions, fragment ions, cone voltage, dwell time and collision voltage were optimized for quantification (Table I). Loss of carboxylic acid group $(M–H–44)^-$ was detected for most of the phenolic acids after collision-induced fragmentation, i.e., m/z 169 > 125 for gallic acid, m/z 153 > 109 for protocatechuic acid, m/z 179 > 135 for caffeic acid, m/z 163 > 119 for *p*-coumaric acid. Fragmentation of some phenolics produced an anion radical $(M–H–15)^-$ by losing a methyl group from the precursor ion besides carboxylic acid group, i.e., m/z 197 > 182 for syringic acid, m/z 223 > 208 for sinapic acid, m/z 193 > 178 for ferulic acid. The precursor ion at m/z 609 eluting at 6.27 min was found. Rutin produced its aglycone, quercetin, $(M–H–309)^-$ by losing a rutinose group. Hesperetin (m/z 302) was produced by collision-induced fragmentation of hesperidin, $(M–H)^-$ at m/z 609. Rosmarinic acid was identified at retention time of 6.93 min. MS fragmentation of precursor ion $(M–H)^-$ in the negative mode at 359 m/z showed three ion fragments at 197, 161, 135 m/z. Quercetin, naringenin, morin, kaempferol and apigenin has similar fragment ions due to their flavone structure, i.e., m/z 301 > 150.9 for quercetin, m/z 271 > 150.9 for naringenin, m/z 301 > 150.9 for morin, m/z 285 > 150.9 for kaempferol and m/z 269 > 150.9 for apigenin.

Method validation

System suitability parameters were determined. The proposed method was validated with good precision (RSD% 0.54–2.72 for intra-day, 1.71–4.64 for inter-day), reproducibility (as percentage recovery: REC% 92–109) and linearity (r = 0.9988–0.9999) (Table II). LOD and LOQ values were calculated based on the critical S/N previously described with six replicates (n = 6). Limits of detection ranged from 0.02 to 18.2 ng/mL. The linear responses of the tested compounds were measured between 1.5 and 6,100 ng/mL to cover a relatively wide linear concentration range.

Phenolic profile of herbal extracts

The content of phenolic compounds detected in the extracts of aromatic *Lamiaceae* species (*Mentha pulegium* L., *Origanum majorana* L. and *Lavandula officinalis*) was determined by UPLC-DAD-ESI-MS/MS system. Total ion chromatograms (TICs) of phenolics identified in *Origanum majorana* L. (Figure 2), *Mentha pulegium L.* (Figure 3) and *Lavandula officinalis* (Figure 4) extracts were shown. Peak identification was carried out by comparing retention times, UV-vis and mass spectra of individual components with those of standards and literature data. The chromatographic findings expressed in milligram quantity per 100 g (mg/100 g) of dry plant sample were shown in Table III. Rosmarinic acid (as the major constituent) was found in higher amounts as compared to those of other phenolic compounds in all species possessing high antioxidant capacity. It was found to be 791 ± 20 mg/100 g in *Mentha pulegium* L., 1,034 ± 26 mg/100 g in *Origanum majorana* L. and 473 ± 3 mg/100 g in *Lavandula officinalis* as major component. Rosmarinic acid is the major antioxidant component in the *Lamiaceae* family also containing hydrocaffeic and caffeic acids (34). Phenolic acids such as caffeic acid (16.6 ± 0.2 mg/100 g), syringic acid (5.64 ± 0.13 mg/100 g) and protocatechuic acid (5.36 ± 0.03 mg/100 g) were detected in distinct amounts in *Mentha pulegium* L. Among flavonoids, hesperidin (73.0 ± 0.16 mg/100 g), apigenin (2.92 ± 0.03 mg/100 g), rutin (1.80 ± 0.02 mg/100 g), naringenin (0.58 ± 0.04 mg/100 g) and quercetin (0.38 ± 0.02 mg/100 g) were found in *Mentha pulegium* L. Apigenin (31.9 ± 0.7 mg/100 g), caffeic acid (24.8 ± 0.3 mg/100 g) and rutin (10.5 ± 0.2 mg/100 g) were detected in *Origanum majorana* L. extracts as secondary constituents in addition to the major one, rosmarinic acid.

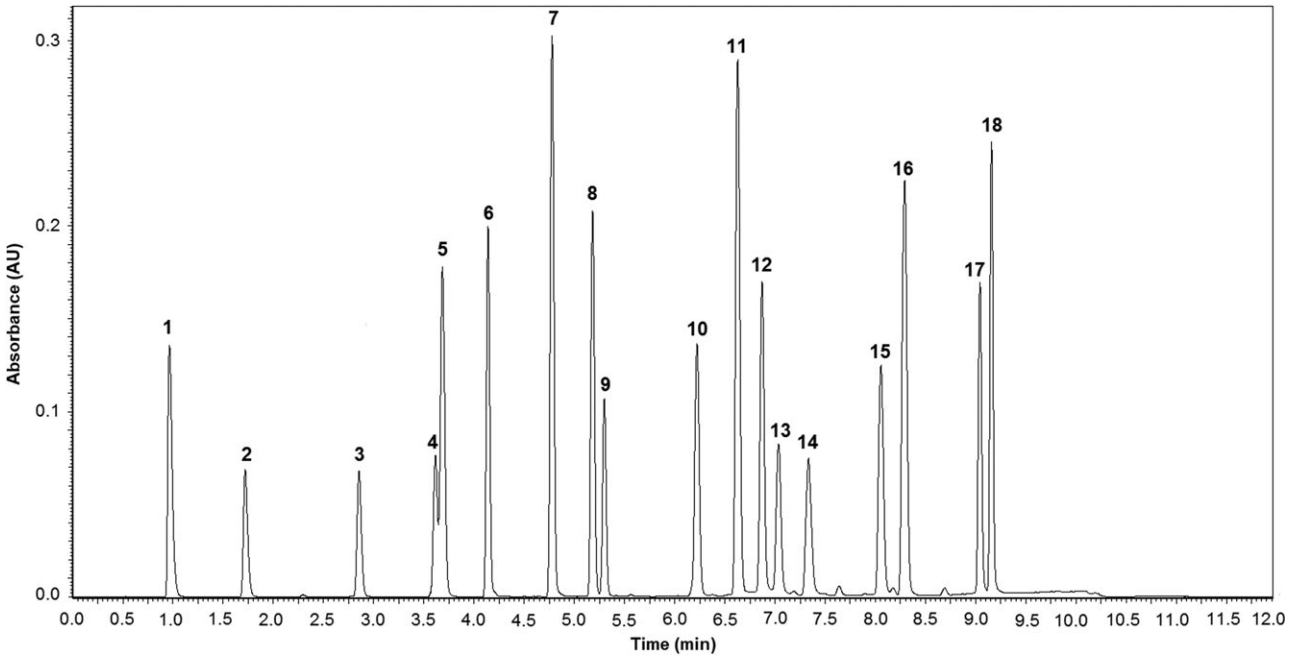


Figure 1. UPLC-PDA chromatogram of phenolic compounds (C_{final}: 50 μM) detected at 280 nm in standard mixture solution; Peak designations are (1) Gallic acid, (2) Protocatechuic acid, (3) Catechin, (4) Vanillic acid, (5) Caffeic acid, (6) Syringic acid (7) p-Coumaric acid, (8) Ferulic acid, (9) Sinapic acid, (10) Rutin, (11) Hesperidin, (12) Rosmarinic acid, (13) Fisetin, (14) Morin, (15) Quercetin, (16) Naringenin, (17) Kaempferol, (18) Apigenin.

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Table I. Optimal Conditions for MRM Transitions of Phenolic Compounds

Compound	t_R (min)	MRM (m/z)	Fragments	Dwell time	Cone (V)	Collision (V)
Gallic acid	1.04 ± 0.01[a]	168.96[b] > 124.95[c]		0.02	36	14
Protocatechuic acid	1.79 ± 0.02	152.95 > 108.94		0.02	35	14
Catechin	2.92 ± 0.04	289.06 > 245.09	203.09 (44)	0.02	38	14
			108.96 (59)			
Vanillic acid	3.68 ± 0.03	166.99 > 107.98		0.02	32	18
Caffeic acid	3.75 ± 0.03	178.97 > 135.00		0.02	32	18
Syringic acid	4.19 ± 0.02	197.03 > 181.99	122.94 (78)	0.02	30	16
p-Coumaric acid	4.84 ± 0.02	162.97 > 119.49		0.02	25	16
Ferulic acid	5.24 ± 0.02	193.03 > 134.01	178.05 (52)	0.02	32	15
Sinapic acid	5.35 ± 0.02	223.00 > 208.03	163.99 (61)	0.02	34	14
Rutin	6.27 ± 0.02	609.27 > 300.19	271.06 (42)	0.02	65	38
Hesperidin	6.68 ± 0.02	609.28 > 301.10	164.02 (12)	0.02	56	26
Rosmarinic acid	6.93 ± 0.02	359.09 > 160.97	197.00 (59)	0.02	46	22
			135.00 (21)			
Fisetin	7.10 ± 0.02	285.08 > 134.98		0.02	52	22
Morin	7.39 ± 0.02	301.06 > 150.98	124.98 (70)	0.02	42	20
Quercetin	8.12 ± 0.01	301.23 > 150.95		0.02	30	26
Naringenin	8.36 ± 0.02	271.05 > 150.98		0.02	54	16
Kaempferol	9.09 ± 0.01	285.04 > 92.90	150.96 (58)	0.02	48	19
Apigenin	9.22 ± 0.01	269.07 > 116.99	150.98 (35)	0.02	54	35

Relative abundance (RA%) given in paranthesis was taken as 100 for product ion.
[a]Values are (mean ± SD) ($n = 6$) of inter-day assays.
[b]Precursor ion $(M–H)^-$.
[c]Product ion.

Table II. Linearity, LOD, LOQ, Precision and Accuracy Results for Tested Compounds

Compound	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)	Calibration equation[d]	Correlation coefficient (r)	RSD%[a] (intra-day)	RSD%[b] (inter-day)	REC%[c]
Gallic acid	1.00	3.30	9.4–1,880	$y = 9.90x – 22$	0.9997	1.10	2.29	102.9
Protocatechuic acid	0.20	0.66	1.5–1,540	$y = 12.09x + 4$	0.9992	1.20	3.22	97.4
Catechin	1.60	5.30	14.5–2,900	$y = 3.35x + 12$	0.9998	1.02	3.58	109.0
Vanillic acid	1.73	5.71	8.4–1,680	$y = 1.04x – 4$	0.9988	0.54	4.02	96.3
Caffeic acid	0.61	2.02	9–1,800	$y = 17.65x + 24$	0.9998	1.24	1.93	107.0
Syringic acid	0.47	1.54	2–2,000	$y = 1.93x + 2$	0.9994	1.37	3.00	99.0
p-Coumaric acid	1.96	6.47	16.4–1,640	$y = 2.46x + 11$	0.9999	1.17	3.83	96.3
Ferulic acid	0.25	0.83	1.9–1,940	$y = 7.20x + 4$	0.9999	0.81	4.10	98.1
Sinapic acid	0.07	0.22	2.2–2,240	$y = 6.62x + 1$	0.9999	0.92	2.26	100.9
Rutin	0.50	1.66	6.1–6,100	$y = 11.66x + 13$	0.9999	0.82	1.93	99.0
Hesperidin	1.17	3.86	30–6,100	$y = 10.76x – 28$	0.9999	0.78	3.31	92.0
Rosmarinic acid	1.43	4.73	7.2–3,600	$y = 3.14x – 10$	0.9993	1.98	3.87	99.0
Fisetin	0.02	0.06	2.9–2,800	$y = 5.39x + 2$	0.9989	1.30	4.14	97.1
Morin	1.42	4.68	15.1–3,000	$y = 2.22x – 7$	0.9996	1.00	2.59	106.0
Quercetin	0.08	0.25	3.4–3,300	$y = 5.88x + 1$	0.9990	1.80	3.00	104.5
Naringenin	1.07	3.54	13–2,720	$y = 20.14x + 48$	0.9990	2.29	2.65	101.5
Kaempferol	2.15	7.10	14.5–2,860	$y = 2.09x + 10$	0.9993	2.72	4.64	101.0
Apigenin	5.52	18.20	27–2,700	$y = 6.77x – 83$	0.9989	0.68	1.71	100.5

[a]Values are means of intra-day assays ($n = 6$).
[b]Values are means of inter-day assays ($n = 6$).
[c]($n = 3$).
[d]Chromatographic peak area (denoted by the symbol y) versus concentration (denoted as x) where x was in ng mL^{-1}.

TAC and phenolic content

The extraction of plants was performed using MAE technique with various solvents (methanol, ethanol, acetone) at different volume ratios (80% and 60% (v/v)). Antioxidant capacities of these extracts were compared to each other to choose the most suitable solvent medium. According to the findings, 60% (v/v) methanol was selected as optimal solvent to achieve maximal extraction efficiency (Figure 5).

The TAC values of microwave-assisted plant extracts were evaluated by the conventional CUPRAC assay (30) and TPC by Folin–Ciocalteau assay (31). The TAC results expressed in "mmol trolox per gram" equivalents were depicted in Table IV. TAC of *Mentha pulegium* L.,

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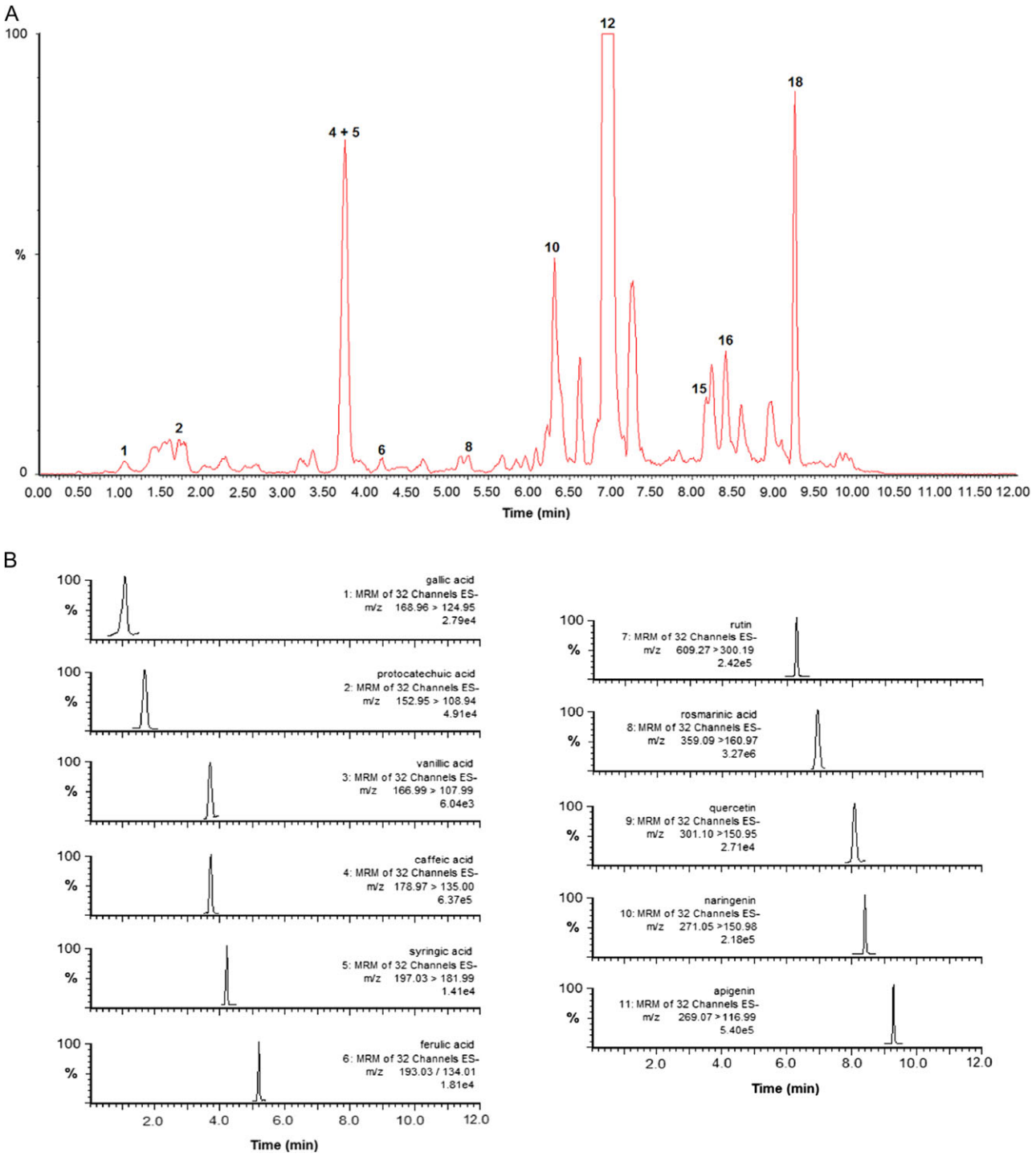


Figure 2. Total (A) and extracted (B) ion chromatograms of phenolics in *Origanum Majorana* L. extract.

Origanum majorana L. and *Lavandula officinalis* extracts were found as 0.58 ± 0.01, 0.66 ± 0.03 and 0.39 ± 0.01 mmol TR/g, respectively. TPC in aqueous methanolic extract of *Mentha pulegium* L. was found as 85.1 ± 2.8 mg GAE/g, whereas those of *Origanum majorana* L. 119 ± 3.4 mg GAE/g, and of *Lavandula officinalis* 57.8 ± 2.1 mg GAE/g. The highest TAC was observed in *Origanum majorana* L. (0.66 ± 0.03 mmol TR/g) due to its higher phenolic content. This order was found in accordance with the findings of the CUPRAC assay.

DPPH free radical scavenging activity of plant extracts

In order to test the free radical scavenging potency of phenolic fractions of *Mentha pulegium* L., *Origanum majorana* L. and *Lavandula officinalis*, the DPPH radical scavenging activity assay (33) was applied. The concentration of plant extract necessary to decrease the initial DPPH radical concentration by 50% (IC_{50}) is widely used to estimate the scavenging ability. The IC_{50} values were found in the following order: *Origanum majorana* L. (67.5 ± 1.2 µg/mL),

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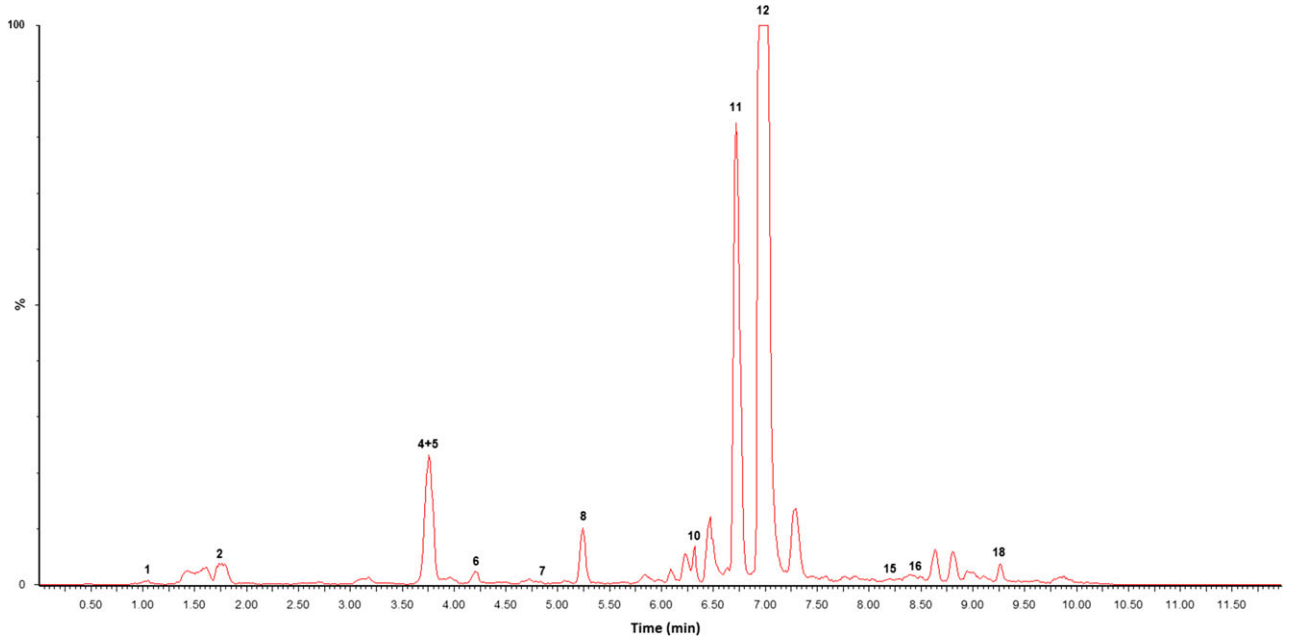


Figure 3. TICs of phenolics identified in *Mentha pulegium* L. extract.

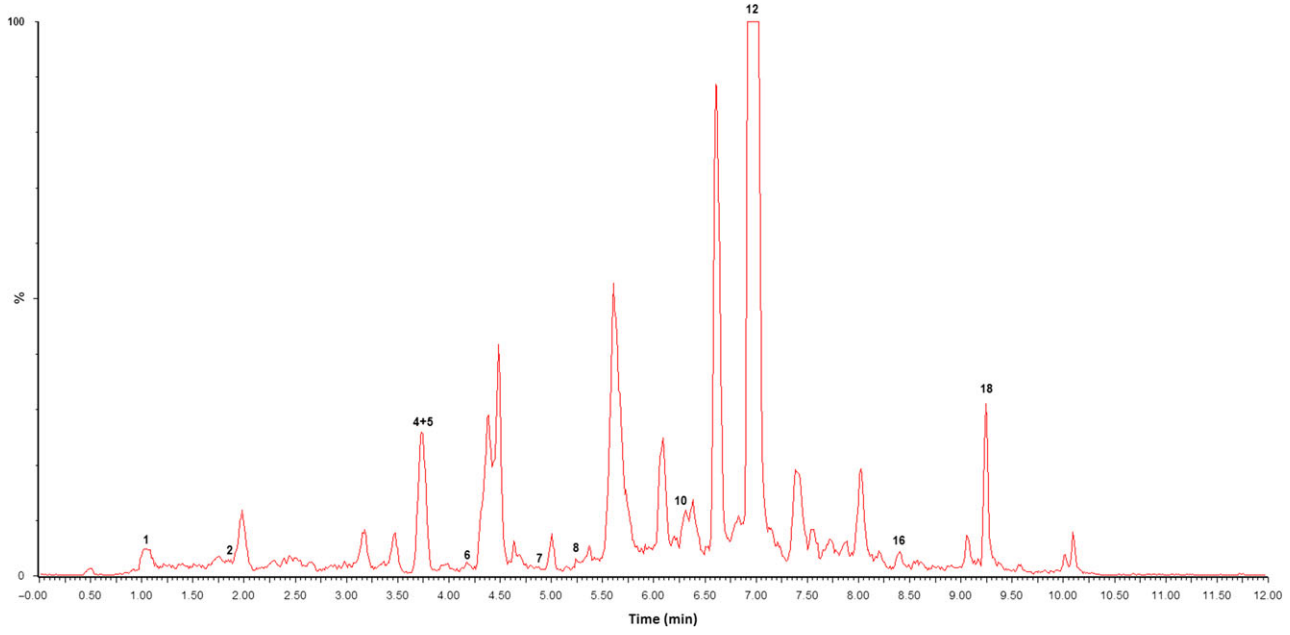


Figure 4. TICs of phenolics identified in *Lavandula officinalis* extract.

Mentha pulegium L. (73.5 ± 2.1 µg/mL) and *Lavandula officinalis* (125 ± 4.6 µg/mL) (Table IV). The highest DPPH radical scavenging activity was found in *Origanum majorana* L. extracts. This order was in accordance with those measured by CUPRAC and Folin–Ciocalteau assays.

Discussion

In this study, a chromatographic method was developed for screening the phenolic composition of pennyroyal (*Mentha pulegium* L.), marjoram (*Origanum majorana* L.) and lavender (*Lavandula officinalis*) extracts on the basis of results of an UPLC system equipped with DAD and MS/MS detectors. ESI negative mode was found to be more sensitive for phenolics according to previous studies (17, 35). The MS/MS parameters such as MRM transitions (Table I) were found relevant with those of previously reported (35, 36). Eighteen compounds were separated within 12 min and showed good reproducibility, ranging from 92% to 109% recoveries with high linearity ($r \geq 0.9988$). Limits of detection values of polyphenols extended down to 0.02 ng/mL. These experimental findings indicated that the UPLC assay coupled with ESI MS/MS provides lower limits of detection and quantification and improved resolution with

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Table III. Quantitative Analysis of Phenolic Compounds Identified in Herbal Plant Extracts (mg/100 g dw)

Phenolic Compounds	*Mentha pulegium* L. (mg/100 g)	*Origanum majorana* L. (mg/100 g)	*Lavandula officinalis* (mg/100 g)
Gallic acid	0.83 ± 0.02	0.34 ± 0.08	0.30 ± 0.03
Protocatechuic acid	5.36 ± 0.03	4.56 ± 0.04	0.99 ± 0.03
Catechin	ND	ND	ND
Vanillic acid	1.54 ± 0.02	1.37 ± 0.06	0.47 ± 0.04
Caffeic acid	16.6 ± 0.2	24.8 ± 0.3	4.05 ± 0.05
Syringic acid	5.64 ± 0.13	3.12 ± 0.09	0.64 ± 0.04
p-Coumaric acid	1.51 ± 0.28	ND	0.35 ± 0.02
Ferulic acid	0.51 ± 0.01	0.97 ± 0.05	0.36 ± 0.01
Sinapic acid	ND	ND	ND
Rutin	1.80 ± 0.02	10.5 ± 0.2	0.66 ± 0.05
Hesperidin	73.0 ± 0.16	ND	ND
Rosmarinic acid	791 ± 20	1,034 ± 26	473 ± 3
Fisetin	ND	ND	ND
Morin	ND	ND	ND
Quercetin	0.38 ± 0.02	3.81 ± 0.16	ND
Naringenin	0.58 ± 0.04	5.43 ± 0.10	0.36 ± 0.01
Kaempferol	ND	ND	ND
Apigenin	2.92 ± 0.03	31.9 ± 0.7	5.35 ± 0.30

Values are (mean ± SD) ($n = 3$).
ND, not detected.

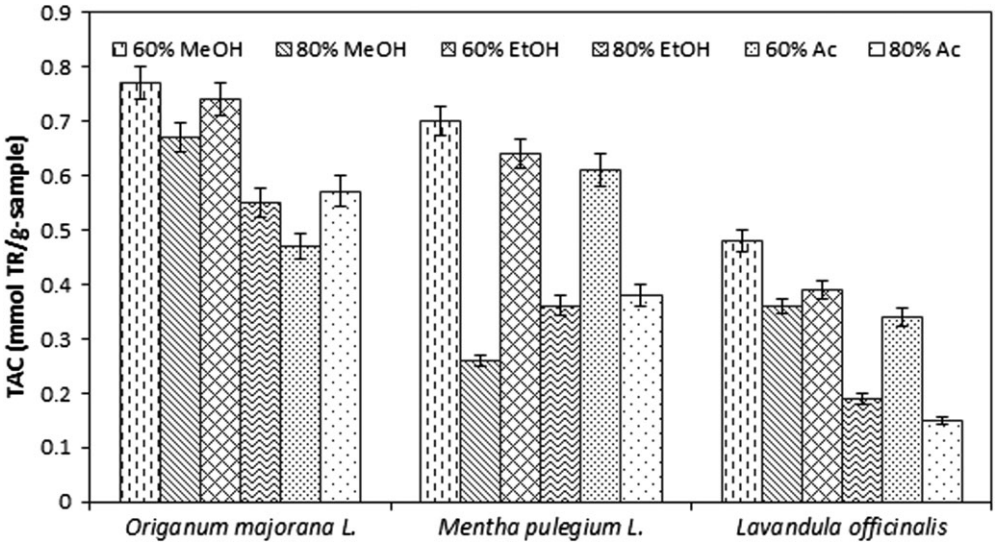


Figure 5. Effect of solvent type on the TAC of plant extracts with respect to the CUPRAC assay.

Table IV. TAC (mmol TR/g), TPC (mg GAE/g) and IC_{50} Values (μg/mL) of Herbal Plant Extracts

Samples	TAC (mmol TR/g)	TPC (mg GAE/g)	IC_{50}[a]
Origanum majorana L.	0.66 ± 0.03	119 ± 3.4	67.5 ± 1.2
Mentha pulegium L.	0.58 ± 0.01	85.1 ± 2.8	73.5 ± 2.1
Lavandula officinalis	0.39 ± 0.01	57.8 ± 2.1	125 ± 4.6

Values are (mean ± SD) ($n = 3$).
[a]IC_{50} (μg/mL): Inhibition concentration at which 50% of DPPH radical is scavenged.

less analysis time compared to the conventional chromatographic assays (4, 35, 37).

As suitable solvent, 60% (v/v) methanol was selected because MeOH is the alcohol that best supports phenolics ionization facilitating oxidation to aryloxy radicals (38). Both the rate and efficiency of phenols oxidation is enhanced by deprotonation of phenols to phenoxide anions. Water at optimal ratio should be present in the solvent medium to solubilize the hydrophilic antioxidants.

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The proportion of water in solvent media is expected to increase the extraction efficiency by expansion of the contact surface area (39). Proestos and Komaitis (40) stated that 60% (v/v) methanol is the best choice to extract polyphenols from plant tissues. The optimal percentage of 60% MeOH in the extracting solvent was also found the best choice for other similar studies (41, 42).

As for the phenolic profile of herbal extracts, our results were generally in good conformity to those of other researchers. For example, apigenin, naringenin, caffeic acid and ferulic acid were detected in high amounts in the methanolic extracts of *Mentha pulegium* L. leaves by HPLC-UV detection according to the results given by Proestos *et al.* (4). We detected high amounts of rosmarinic acid (i.e., found as the most abundant phenolic constituent), apigenin and caffeic acid in *Lavandula officinalis*. Benzoic acid and cinnamic acid derivatives were found at low concentrations. Our research generally revealed similar phenolic content with previous studies (6, 15). Kovatcheva-Apostolova *et al.* (43) did not detect catechin in *Lavandula vera* in accordance with our findings. In contrast to our results, Proestos *et al.* (4) and Taamalli *et al.* (14) detected catechin in these plant extracts.

TAC, TPC and DPPH free radical scavenging activity of the extracts were determined. TPC (expressed as mg gallic acid/g of dry sample) order reported by Proestos *et al.* (4) was similar to our findings. According to their results, *Origanum majorana* L. showed the highest phenolic content among others. Our free radical scavenging, CUPRAC and Folin–Ciocalteau assay results were consistent with the findings of Zugic *et al.* (7) and Roby *et al.* (37) in terms of the correlation between radical scavenging activity and TAC/phenolic content. Although the overall mechanisms of the concerned assays may be different, free radical scavenging activity can be related to phenolic content and phenolic composition of plant extracts.

TAC (CUPRAC), TPC (Folin–Ciocalteau) and IC_{50} (DPPH) results of the three plant extracts correlated well among each other. There may not be a one-to-one correspondence between the results of different antioxidant capacity assays (i.e., the results may not be identical or too close), because each assay has its own reagent with varying oxidation potential, reaction kinetics, solvent dependency, etc. (44). The order of reported CUPRAC antioxidant capacities as trolox equivalents in Table IV was *Origanum majorana* L. > *Mentha pulegium* L. > and *Lavandula officinalis*. The same order was obtained by Folin–Ciocalteau and DPPH assays. These findings revealed that *Origanum majorana* L. showed the greatest capacity, due to its higher phenolic content. The overall findings of the present study indicate that microwave-assisted aqueous methanolic extracts of Lamiaceae species, namely *Mentha pulegium* L., *Origanum majorana* L. and *Lavandula officinalis*, are rich in phenolic antioxidants. These results reveal that Lamiaceae family herbal plants may be good supplements to help protect cells against oxidative damage caused by free radicals.

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