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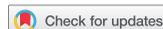
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Phenolic profile and biological activities of *Micromeria graeca* (L.) Benth. ex Rchb.

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ABSTRACT

The medicinal potential of the ethanol extract of *Micromeria graeca* (L.) Benth. ex Rchb., harvested in Algeria (MG extract), was evaluated by assessing *in vitro* its antioxidant, antibacterial, and antityrosinase properties. The total phenolic (= 430 ± 30 mg gallic acid equivalent/100 g of dry weight) and flavonoid (= 190 ± 10 mg quercetin equivalent/100 g of dry weight) contents were evaluated by the Folin–Ciocalteu and the aluminium chloride methods, respectively. Silica gel thin-layer chromatography revealed the presence of chlorogenic, caffeic, and rosmarinic acids and diosmin; the presence of these compounds was confirmed by reverse phase high-performance liquid chromatography. The IC₅₀ values indicate that the ethanol extract of *M. graeca* is highly potent in neutralizing the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation radicals (ABTS^{•+}; IC₅₀ = 30.5 ± 0.9 µg/mL) and the 2,2'-diphenyl-1-picrylhydrazyl radicals (DPPH[•]; IC₅₀ = 65.8 ± 2.4 µg/mL). The MG extract was also very active on a lipid peroxidation model (IC₅₀ = 23.4 ± 1.9 µg/mL). The superoxide quenching index values (electrochemically measured) were 332 ± 37 (Al₃₀) and 638 ± 53 µg/mL (Al₅₀). The studied extract showed a moderate tyrosinase inhibitory activity (IC₅₀ = 302 ± 62 µg/mL) and a low antibacterial effect while the combination of this extract with antibiotics restored the activities of cefotaxime and streptomycin on resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

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Introduction

Plants of the genus *Micromeria* are perennial herbs belonging to the family Lamiaceae, tribe Mentheae, and subfamily Nepetoideae.^[1] This genus includes about 130 species, widespread in the Mediterranean region.^[2] *Micromeria* is part of a complex group of genera that comprises herbs, subshrubs, and shrubs with medicinal value, used in popular medicine against heart disorders, headache, wound skin, infections, and as antispasmodic, stimulant, expectorant,^[3] and to treat colds.^[4] Furthermore, the leaves of these plants have been reported to possess anti-inflammatory and antimicrobial properties.^[5] The genus *Micromeria* grows naturally in Algeria, where its leaf decoction has been generally used in folk medicine as a substitute for mint, notably in the treatment of wounds, stomach ache, colds, and fevers and as condiment.^[6]

As for many Lamiaceae, the studies on *Micromeria* sp., including *Micromeria graeca*,^[7,8] have mostly concerned their essential oils; nevertheless, previous researches have established the genus *Micromeria* to be a rich source of polyphenolic compounds.^[4,9] Polyphenols are bioactive molecules

attracting high scientific attention. These compounds exhibit a wide range of pharmacological effects that are linked to their antioxidant properties.^[10,11] Phenolic compounds have been reported to be excellent free radical scavengers due to their strong hydrogen-donating ability.^[10,12]

Reviewing the literature, nothing was reported concerning the phenolics of *M. graeca* (L.) Benth. ex Rchb., except a few papers concerning the occurrence of flavonoidal glycosides.^[1,13] For that reason, the objectives of the present study were (i) to analyse the phenolic composition of *M. graeca* (MG) collected from the North-Eastern Bejaia region in Algeria; and (ii) to investigate the anti-bacterial, tyrosinase inhibitory, and antioxidant activities of a polar extract of this plant.

Materials and methods

Plant material

The aerial parts were collected at the flowering stage in Smaoun (latitude: 36°36'N; longitude: 4°49'E) in Bejaia, Northeastern Algeria, August 2009. The plant specimen was further authenticated by Professor Lejoly in the Laboratory of Systematical Botany and Phytosociology, Free University of Brussels (ULB). A voucher specimen (BR 000000 6946197) has been deposited in the Herbarium of the National Botanical Garden of Meise (Belgium). The sample was dried in the shade at room temperature until constant weight and ground in an electric mill to pass a 0.25 mm sieve.

Chemicals

Folin-Ciocalteus's phenol reagent and sodium carbonate were purchased from Biochem Chemopharma (Montreal, Quebec). Gallic acid was purchased from BDH, Prolabo (Linars del Vallés, Spain), rosmarinic acid and kaempferol were purchased from Carl Roth (Karlsruhe, Germany), apigenin-7-glucoside, apigenin, chlorogenic acid, ellagic acid, diosmin, epicatechin, and naringenin from Sigma-Aldrich (St. Louis, USA), caffeic acid from Janssen Chimica (Beerse, Belgium), ferulic acid from Fluka (Buchs, Switzerland), rutin from Alfa Aesar (Ward Hill, MA), and quercetin from Riedel-de Haen (Germany). All standards were prepared as stock solutions in methanol (1 mg/mL). Mushroom tyrosinase (5.370 U/mg), L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), and kojic acid were purchased from Sigma-Aldrich (St. Louis, USA); ABTS^{•+} and DPPH[•], *N,N*-dimethylformamide extra dry ($\leq 0.005\%$ H₂O) and tetrabutyl ammonium hexafluorophosphate (electrochemical grade) from Fluka (Buchs, Switzerland); Trolox[•] [(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] from Acros Organics (Morris Plains, NJ, USA). The Mueller-Hinton broth was purchased from Oxoid (Hampshire, UK). Cefotaxime and streptomycin were used as control for the microdilution assays (Taastrup, Denmark). All other reagents were obtained from Sigma-Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany) and were of analytical grade.

Preparation of plant extract

The aerial parts powder (25 g) was first defatted with 600 mL *n*-hexane, and then extracted by stirring with 400 mL of ethanol, at room temperature (130 rpm, 24 h). The crude extract was filtered. The filtrate was concentrated with a rotary vacuum evaporator, lyophilized, and kept in the dark at +4°C until tested. The extraction yield was 4.8%.

Estimation of the content in phenolic compounds

Total phenolics content

Total phenolic content (TPC) was estimated as gallic acid equivalents according to the Folin-Ciocalteu method as described in our previous study.^[14] First, 100 μ L of sample (1 mg/mL) or gallic

acid (1–10 µg/mL) were transferred to a 10.0 mL volumetric flask containing 6.0 mL of H₂O, to which were subsequently added 500 µL of undiluted Folin–Ciocalteu reagent. After 1 min, 1.5 mL of 20% aqueous Na₂CO₃ was added, and the volume was made up to 10.0 mL with H₂O. The control contained all the reagents except the extract. After 2 h incubation at 25°C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. TPC was determined as milligram gallic acid equivalent per 100 g of dry matter (mg GAE/100 g DM).

Total flavonoids content

Total flavonoids content (TFC) was measured by a colorimetric assay as reported in our previous study.^[14] 1.5 mL of diluted sample (250 µg/mL) or standard solution (5–25 µg/mL) was added to 1.5 mL of AlCl₃ solution (2%) and mixed. After 15 min, the absorbance of the mixture was determined at 430 nm against the blank. TFC was expressed as milligram quercetin equivalent per 100 g of dry matter (mg QE/100 g DM), based on the quercetin calibration curve.

Determination of antioxidant activity

Inhibition of linoleic acid peroxidation

The inhibition of the Fenton reaction-induced linoleic acid peroxidation was measured by an indirect *in vitro* colorimetric method.^[15] The reaction mixture contained 125 µL phosphate buffer (0.4 M, pH 6.75, air-saturated), 25 µL of extract solutions (serial dilutions ½ in methanol; initial concentration, 10 mg/mL), 10 µL of hydrogen peroxide (16 mM), 10 µL of ferrous sulfate (16 mM)–EDTANa₂H₂ (15 mM), and 80 µL of linoleic acid (8 mM in 50 mM borate, nitrogen-saturated, pH 9). After 10 min at room temperature in the dark, 75 µL of each reaction well were transferred to another plate containing 150 µL of a benzoyl leucomethylene blue–haemoglobin solution (5 mg benzoyl leucomethylene blue, in 8 mL dimethyl formamide, added to 80 mL phosphate buffer, 0.2 M, pH 5, containing 1.4 g Triton X-100 and 5.6 mg of haemoglobin; diluted to 100 mL with phosphate buffer; to be prepared extemporaneously, strictly observing the order of reagents addition) per well. After 30 min at room temperature in the dark, the absorbance was measured with a Labsystems EMS reader/dispenser MF (Labsystems, Finland) at 660 nm. All the experimental results obtained from two separate experiments in triplicate were fitted to a parametric function in Systat 7.1 (Systat Software) as described in Fig. 1. The IC₅₀ (the concentrations of plant extract that inhibit 50% of methylene blue formation) were computed. Results were expressed as Trolox[°] equivalent antioxidant capacity (TEAC), calculated as IC₅₀Trolox[°]/IC₅₀ plant extract.

Quenching of the ABTS^{•+} radical cation

The TEAC was performed using discoloration assay.^[14] ABTS^{•+} radical cation was produced by reacting ABTS stock solution of 7 and 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The solution was then diluted to obtain an absorbance of 0.70 ± 0.02 units at 734 nm. Ten microliters of extract (10–60 µg/mL) were added to 990 µL of ABTS^{•+} solution, and the decrease in absorbance at 734 nm was monitored for 30 min. The TEAC of gallic acid (2–6 mM) and quercetin (5–10 mM) samples were also assayed for comparison. A concentration–response curve as a function of different Trolox[°] concentrations (5–20 µM) was used for calculating the TEAC. All determinations were performed in triplicate. The percentage inhibition was calculated as:

$$\text{ABTS}^{\bullet+} \text{ radical scavenging activity (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{(\text{Abs}_{\text{control}})} \right] * 100$$

where Abs_{control} is the absorbance of ABTS^{•+} + ethanol and Abs_{sample} is the absorbance of ABTS^{•+} + sample extract/standard.

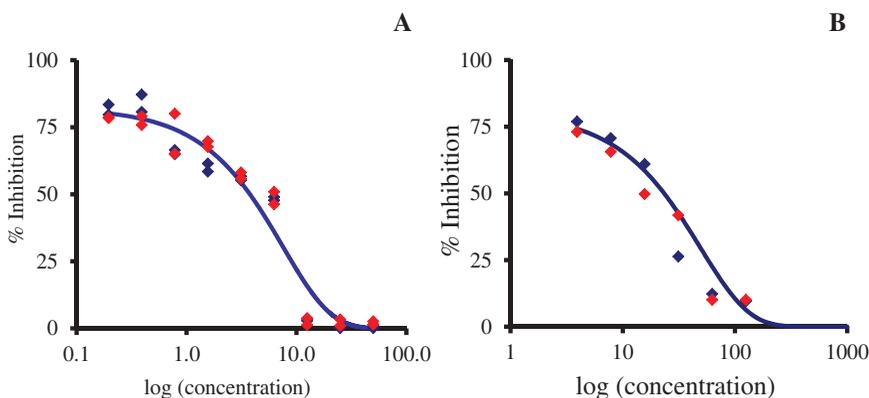


Figure 1. Lipid peroxidation model of *M. graeca* extract. The experimental results obtained from two separate experiments in triplicate were fitted to a parametric function in Systat 7.1 (Systat Software), $N = N^0 \times \exp(-k \times C)$, where C is the concentration, N is the percentage of linoleic acid remaining at concentration C , N^0 is the percentage of linoleic acid at concentration 0, and k is the parameter. Trolox[®] was used as a reference. ◆ Plate 1 ; ◆ plate 2 ; (A) Trolox[®] (B) *M. graeca* extract.

Quenching of the DPPH[•] radical

The DPPH[•] radical-scavenging activity was measured using the technique described in our previous study.^[14] 0.5 mL of the DPPH[•] solution (1.10^{-3} M) was added to 1 mL of plant extract (10–80 $\mu\text{g}/\text{mL}$) or 1 mL of ethanol. The mixture was incubated at room temperature in the dark. The decrease in absorbance of the resulting solution was monitored at 517 nm at 60 min. The scavenging activity was estimated based on the percentage of DPPH[•] radical scavenged as the following equation:

$$\text{Scavenging effect(\%)} = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} \times 100.$$

The IC_{50} value is the effective concentration that could scavenge 50% of the DPPH[•] radicals. Gallic acid (10–80 $\mu\text{g}/\text{mL}$), quercetin (0.5–2.5 $\mu\text{g}/\text{mL}$), and Trolox[•] (2.0–8.0 $\mu\text{g}/\text{mL}$) standards were used as positive references.

Quenching of electrochemically generated superoxide anion

Cyclic voltammetry experiments were performed on dual potentiogalvanostat PGSTAT 100 (Autolab instrument, Eco Chemie B.V., Utrecht, The Netherlands). All measurements were carried out on a three-electrode thermostated cell. A glassy carbon disk working electrode (diameter 2 mm), a platinum wire counter electrode, and a reference electrode, Ag/AgCl in EtOH saturated by LiCl were used for all electrochemical experiments. The reference electrode was separated from the solution by a salt bridge containing 0.5 M Bu_4NPF_6 in *N,N*-dimethylformamide (DMF). The glassy carbon disk working electrode was polished using silicon carbide 4000 paper with a laboPol-5 (Struers, Ballerup, Denmark), washed with distilled water, and then dried. For all measurements, the temperature was maintained at $20 \pm 0.02^\circ\text{C}$ with a Julabo heating circulator MP-5 (Julabo, Seelbach, Germany).

A voltammetric technique was used to generate $\text{O}_2^{\bullet-}$ by reduction of molecular oxygen in an aprotic medium (DMF extra dry ($\leq 0.01\% \text{H}_2\text{O}$)). The reactivity of the radical $\text{O}_2^{\bullet-}$ was directly measured in cyclic voltammetry at the backward scan by the anodic current decay from its oxidation in the presence of extract.

A solution of 10 mL of an extra dry DMF containing the supporting electrolyte 0.1 M Bu_4NPF_6 was saturated by dry air during 10 min. In these conditions, the solubility of oxygen was assumed to be $C_{\text{O}_2} \approx 9.4 \times 10^{-4}$ mol/L, this value corresponding to a partial pressure of 0.2 bar.^[14] The cyclic voltammogram (CV) of the oxygen reduction was then recorded at a scan rate 0.1 V/s, with the initial potential at 0 V and the reverse one at -1.5 V vs. Ag/AgCl.

Stock solution of the plant extract was prepared at about 5 mg/mL. Aliquots of stock solution were successively added to the 10 mL oxygen solution in order to get a plant extract concentration in the range of 50–600 µg/mL. The CV of oxygen without phenolic compounds or extract is first recorded in order to get the initial cathodic and anodic peak currents. Then, successive aliquot additions are performed and CV recorded at a scan rate of 0.1 V/s after each aliquot addition.

Inhibition of tyrosinase activity

The tyrosinase inhibitory activity of the extract was evaluated by using L-DOPA.^[14,16] In a 96-well microplate, 80 µL of the sample (50, 100, and 500 µg/mL) or kojic acid (142 and 1421 µg/mL) dissolved in 20 mM phosphate buffer, pH 6.8, were rapidly added with 90 µL of mushroom tyrosinase (45 units/mL, dissolved in the pH 6.8 buffer). After the rapid addition of 80 µL of a mixture of L-DOPA/L-tyrosine in the same buffer and incubation at 37°C for 5 min, the absorbances were measured at 450 nm every 20 min over 180 min with a Multi-Mode Microplate Reader (Multiskan Ex., USA). The following equation was used to determine the inhibition of the tyrosinase activity.

$$\% \text{ inhibition} = \frac{A_{\text{sample}} - A_{\text{negat. control}}}{A_{\text{negat. control}}} \times 100$$

Determination of the antibacterial activity

The antibacterial activity was screened against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 obtained from the American Type Culture Collection, and *S. aureus* C 100459, a methicillin-resistant *S. aureus* (MRSA) clinical isolate, a generous gift from the Centre Hospitalier Universitaire of Charleroi, Belgium.

The direct antibacterial effect was evaluated by a broth microdilution method according to Okusa et al.,^[17] the plant extract was dissolved in DMSO (20 mg/250 µL) and diluted to 5 mL with Mueller–Hinton broth, the final DMSO concentration being 5%. This solution was transferred in 96-well plates (200 µL/well) and serially diluted with Mueller–Hinton broth. Bacterial strains cultures were stirred with 0.9% NaCl to achieve 0.5 McFarland (10^8 cells/mL), diluted 1/100 to achieve 10^6 cells/mL, and inoculated in the 96-well plates (100 µL/well). The cultures were incubated at 37°C for 24 h in an oven. The minimum inhibitory concentration (MIC) was the lowest concentration of extract that completely inhibited the growth of microorganisms in the microdilution wells, detected by the unaided eye; the minimal bactericidal concentration (MBC) was determined by sub-culturing the negative wells on a Mueller–Hinton agar plate as the lowest concentration that yielded negative sub-cultures. Controls were set up with streptomycin and cefotaxime.

The analysis of antibacterial combinations analysis was performed according to Okusa et al.^[17] This requires experimental data for agents used alone and in different dose combinations at equieffective levels. The strains *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 9027 were used to test the combination of antibiotics and MG extract. Compounds were placed into 96-well tissue culture plate to obtain mixtures covering a broad range of suboptimal concentrations of both compounds.

Identification of phenolic compounds

TLC analysis

The qualitative investigation of the MG extract with regards to the presence of flavonoids and phenolic acids was carried out on Silica gel 60 F254 plates 0.25 mm (Merck, Germany), using ethyl acetate–formic acid–water (90:6:6, v/v/v) as a solvent system. After spraying the developed plates

with a 1% methanolic solution of diphenyl-boric acid-ethanolamine complex and additionally with a 5% ethanolic solution of PEG 400, the plates were visualized under UV 254/366 and daylight.^[18]

RP-HPLC analysis

Separation and identification of the polyphenolic components in the extract were carried out by HPLC (Kontron), on a silica-based C18-bonded phase column (Alltima 250 × 4.6 i.d. mm; 5 μm; Grace Alltech, Columbia, USA) at 40°C, using UV detector operating at different wavelengths (280 and 330 nm). The injection system used was a 20 μL sample loop. The solvent system comprised mobile phases A (acetonitrile: H₂O, 19:80, v/v with CH₃COOH 1%) and B (acetonitrile: MeOH, 59:40, v/v with CH₃COOH 1%) at a flow rate of 1.2 mL/min. The elution gradient was 0–12% B, 0–8 min; 12–12% B, 8–12 min; 12–45%, 12–20 min; 45–100% B, 20–35 min; 100–0% B, 35–40 min. Reference standards were used for the identification of major phenolic compounds in the sample.

Statistical analysis

The experimental results were given as mean ± SD of three replicate measurements. The experimental values were evaluated by using one-way analyses of variance (ANOVA). *p* values < 0.05 were regarded as 'significant'. The eventual correlations of the radical scavenging (ABTS^{•+}, DPPH[•], and O₂^{•-}) and inhibition (linoleic acid peroxidation and tyrosinase) capacities with total phenolics and flavonoids were also analysed.

Results and discussion

Total phenolic and flavonoid contents

The TPC of Algerian MG is 430 ± 30 mg GAE/100 g dry weight. The most widespread and diverse phenolics are flavonoids, polyphenolic secondary metabolites widely dispersed throughout the plant kingdom. The TFC of MG was 190 ± 10 mg in terms of quercetin equivalents/100 g of dry weight. As far as we know, there are no reports on the TPC and TFC of MG extract.

Vladimir-Knežević et al.,^[19] studying three *Micromeria* species (*Micromeria croatica*, *Micromeria juliana*, and *Micromeria thymifolia*) from Croatia, showed that the total polyphenols in all plant samples were considerable, ranging between 9.7% and 13.1%. The most abundant compounds were phenolic acids (5.3–6.8%), followed by tannins (3.1–6.1%), contrasting with a very low amount of flavonoids (0.01–0.09%).

Antioxidant activity

In complex biological systems, various mechanisms may contribute to oxidative processes, for example, electrons leakages from electron-transport chains, photodynamical effects or Fenton reactions, in which transition metal ions play a major role; different reactive oxygen species (ROS) might be generated targeting various biomolecules, lipids, proteins, and nucleic acids. Depending on their site of generation (aqueous phase or lipid membranes), the disposal of ROS is quite different. Therefore, it is important to characterize the extracts by a variety of antioxidant assays.^[20] To the best of our knowledge, the antioxidant potentialities of MG extract have seldom been studied; Couladis et al.^[21] showed, by a method based on the peroxidation of arachidonic acid, that the methanol extract of MG was similar in activity to α-tocopherol. In the present work, four antioxidant assays were carried out.

Inhibition of linoleic acid peroxidation

Lipid peroxidation can be inhibited by suppressing chain initiation and chain propagation and/or by enhancing chain termination. A haemoglobin-catalysed peroxidation of linoleic acid assay indicated the ability of MG extract to protect unsaturated fatty acids from peroxidation. Curve fitting (Fig. 1) allowed computing IC₅₀ values for the plant extract and Trolox[®] (Table 1); the IC₅₀ value (23.4 ± 1.9 µg/mL) indicates the ability of the plant extract to inhibit lipid peroxidation and corresponds to 0.16 Trolox[®] equivalents. Previous results obtained on *Micromeria nervosa* (Desf.) Benth. extracts and isolated compounds indicate anti-lipid peroxidation activities.^[4] The acetone extract of *Micromeria cilicica* Hausskn. ex P.H. Davis (a synonym of *Clinopodium cilicicum* (Hausskn. ex P.H. Davis) Bräuchler & Heubl.) showed a strong inhibition of lipid peroxidation in a β-carotene bleaching assay, with quasi equivalence to (+)-catechin.^[22] The methanolic extract of *Micromeria fruticosa* ssp. *serpyllifolia* (M. Bieb.) P. H. Davis inhibited linoleic acid oxidation, while the essential oil of this species exhibited practically no activity.^[23]

Quenching of the ABTS^{•+} and DPPH[•] radicals

The ABTS^{•+} and DPPH[•] assays, based on single-electron transfer mechanisms, are simple and common methods for evaluating antioxidant activities.^[20] The IC₅₀ values indicate that the ethanol extract of MG is potent in neutralizing ABTS^{•+} (IC₅₀, 30.5 ± 0.9 µg/mL) and DPPH[•] (IC₅₀, 65.8 ± 2.4 µg/mL) radicals (Table 1). These assays indicate a hydrogen and/or electron donation capacity of the MG polar extract, an important factor to protect biomolecules from ROS in susceptible biological and food systems.

A DPPH[•] radical-scavenging activity was previously recorded for polar extracts of other *Micromeria* species. The acetone extract of *M. nervosa* (from Egypt) quenched DPPH[•] with the same potency as α-tocopherol.^[4] The ethanolic extracts of *M. croatica*, *M. juliana*, and *M. thymifolia* (from Croatia)^[19] and the methanolic extract of *M. fruticosa* ssp. *serpyllifolia* (from Turkey)^[23] exhibited considerable scavenging of DPPH[•]; *M. croatica* was the most active species (IC₅₀ = 4.7 µg/mL). The ethanolic extract of *Micromeria myrtifolia* (from Lebanon) was quite active towards DPPH[•], whereas the essential oils and apolar (hexane and chloroform) extracts showed only marginal antiradical activities.^[3] Vladimir-Knezevic et al.^[19] showed that the radical-scavenging activity of *Micromeria* species largely depends on their major compound, rosmarinic acid, a powerful free antioxidant.

Quenching of the O₂^{•-} radical

This assay for antioxidant capacity, based on the quenching of electrogenerated superoxide, was previously applied to seaweed's, mint, and Ericaceae extracts.^[14,24,25] Biological samples

Table 1. Antioxidant activity and tyrosinase inhibition of *M. graeca* extract and standards.

Sample	Inhibition of linoleic acid peroxidation		DPPH [•] test*		ABTS ^{•+} test		O ₂ ^{•-} quenching		Inhibition of tyrosinase
	IC ₅₀ (µg/mL) †	Trolox [®] equivalent †, §	IC ₅₀ (µg/mL)	IC ₅₀ (µg/mL)	TEAC (mM)	Al ₃₀ (µg/mL)	Al ₅₀ (µg/mL)	IC ₅₀ (µg/mL)	
MG	23.4 ± 1.9	0.16	65.8 ± 2.4 ^d	30.5 ± 0.9 ^f	0.23 ± 0.01 ^d	332 ± 37	638 ± 53	302 ± 62 ^b	
Gallic acid	nd	nd	0.82 ± 0.05 ^a	3.00 ± 0.01 ^a	2.9 ± 0.1 ^a	nd	88 ± 5	nd	
Quercetin	nd	nd	1.96 ± 0.04 ^b	4.06 ± 0.04 ^b	1.95 ± 0.05 ^b	nd	141 ± 6	nd	
Trolox	nd	1	4.6 ± 0.1 ^c	8.8 ± 0.2 ^c	nd	nd	493 ± 17	nd	
Kojik acid	nd	nd	nd	nd	nd	nd	nd	23 ± 1 ^a	

*Data are expressed as mean values ± standard deviation (n = 3). Values within each column with different superscript letters (a, b, c, d, e, f) are significantly different (p < 0.05) as determined using ANOVA.

†Curve fitted from two experiments in duplicate.

§Computed from Trolox[®] IC₅₀ measured along each experiment.

nd: not determined.

are complex mixtures of compounds producing a broad voltammetric wave; the area beneath the major anodic peak correlates with the number of electrons used up in the oxidation reaction and so depends on the quenching capacity of the antioxidant sample.^[26] The measurement of the antioxidant activity was estimated by antioxidant index values AI_{30} and AI_{50} (Table 1), defined as the extract concentrations needed to consume 30% or 50% of the electrogenerated radical, respectively [corresponding to $(I_{p_a^{\circ}} - I_{p_a^S})/I_{p_a^{\circ}} = 0.3$ or 0.5 where $I_{p_a^{\circ}}$ is the intensity of the anodic current peak of $O_2^{\cdot-}$ and $I_{p_a^S}$ is the intensity of the anodic current peak of $O_2^{\cdot-}$ for the concentration S of the sample]. With this characterization, the lower the AI_{30} or AI_{50} value, the more the sample has a strong reactivity towards superoxide (Table 1).

The content of total phenolics correlated with quenching of $ABTS^{\cdot+}$ ($R^2 = 0.996$), superoxide ($R^2 = 0.992$), and lipid peroxidation ($R^2 = 0.977$), but not with $DPPH^{\cdot}$ ($R^2 = 0.093$). By contrast, the content of total flavonoids correlated with quenching of $DPPH^{\cdot}$ ($R^2 = 0.770$) and neither with $ABTS^{\cdot+}$ ($R^2 = 0.059$), superoxide ($R^2 = 0.075$), nor lipid peroxidation ($R^2 = 0.111$). The measured AI_{30} and AI_{50} (Table 1) were considerably higher than the concentrations required to quench $DPPH^{\cdot}$ and $ABTS^{\cdot+}$, indicating a lower antioxidant capacity towards superoxide. In line with previous reports on *Mentha*^[14] and *Erica*^[25] species, these data confirm that there is no correlation between the reactivity towards large stable radicals (neutral or positively charged) and the reactivity towards the small, chemically reactive and negatively charged superoxide radical. Because of their simplicity, methods based on stable radicals are widely used but we agree with the conclusions of Tohaya and Ochiai,^[27] who preconized caution in interpreting such data. Our data corroborate those of Vladimir-Knezevic et al.,^[19] who showed that the obvious antioxidant capabilities of tested *Micromeria* species were not correlated to flavonoids but to phenolic acids, notably rosmarinic acid.

Inhibition of tyrosinase

Tyrosinase is the limiting enzyme in the series of reactions leading to melanin production. As melanin overproduction is observed in many skin disorders, the inhibition of tyrosinase activity would appear to be the most rational and explicit way of overcoming these issues.^[28] Also many skin-whitening agents, mostly based on chemical tyrosinase inhibitors (hydroquinone and kojic acid, notably), are used by African and Asian populations with many adverse and toxic effects; the search for natural tyrosinase inhibitors may lead to safer cosmetics.^[16] Compared to kojic acid, a reference inhibitor (IC_{50} , 8.9 $\mu\text{g/mL}$), the MG extract was found to exhibit a moderate tyrosinase inhibition with IC_{50} value of 302 ± 62 $\mu\text{g/mL}$. As tyrosinase catalyses oxidative reactions,^[11] there should be a close relationship between the antioxidant phenolic compounds of natural extracts and their inhibitory effects.^[28,29] Previous studies have supported the role of metal ion chelation in the process of tyrosinase inhibition, and a linear correlation was found between chelating activity and tyrosinase inhibition.^[30] Polyphenolic compounds, such as flavonoids and acid phenols, are known to form complexes with metal ions to exhibit antityrosinase action.^[29]

Antibacterial activity

The antibacterial activity of the MG extract was tested against four pathogenic bacteria, namely *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538, and *S. aureus* C 100459; Table 2 presents the direct antibacterial effects of the extract, measured by a broth microdilution technique. The extract was inactive against all tested microorganisms ($MIC > 2000$ $\mu\text{g/mL}$). Published data generally indicate that apolar extracts from plants are considerably more antimicrobial than polar extracts. This is often true for plants belonging to the genus *Micromeria*; in fact, the antibacterial activity of these plants is mostly due to their essential

Table 2. The minimal inhibitory concentrations MICs ($\mu\text{g/mL}$) and minimal bactericide concentrations MBCs ($\mu\text{g/mL}$) of *M. graeca* extract and antibiotics.

Sample	<i>S. aureus</i> ATCC 6538		<i>S. aureus</i> C 100459		<i>P. aeruginosa</i> ATCC 9027		<i>E. coli</i> ATCC 25922	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MG extract	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000
Cefotaxime	1	–	4	–	16	–	nd	–
Streptomycin	0.125	–	4	–	>64	–	64	–

nd: not determined.

oils rather than polar compounds. For instance, apolar extracts and essential oils of *M. cilicica* Hausskn. ex P. H. Davis (origin, Turkey) showed antibacterial activity against all of the tested bacteria (agar diffusion test; 10–25 μL essential oils; inhibition diameters, 9–21 mm).^[31] The essential oils from herbal parts of *Micromeria cristata* subsp. Phrygia P.H. Davis collected from three different Turkish localities were active against all pathogenic microorganisms tested (MICs, 31–250 $\mu\text{g/mL}$).^[32] According to Ali-Shtayeh et al.,^[5] the least active extract of *M. nervosa* (Desf.) Benth. (origin, Palestine) was the freeze-dried water extract.

The lack of significant activity of our polar MG extract may be related, at least in part, to hydrophilicity, a factor that may reduce recognition and transport of active compounds. Indeed lipophilicity appears as a key factor for bacterial inhibition, but other factors, such as structural features of the compounds, have to be considered.^[33] According to Rauha et al.,^[34] prenyl-substituted flavanones have been shown to possess more effective antibacterial activity against *S. aureus* than simple flavanones and flavonols; flavonoid glycosides (different glycosides of quercetin), such as found in polar extracts, notably failed to show any activity against the microbial strains tested.^[34] Regarding tannins, which are generally known to present antimicrobial activity, paradoxically some organisms are capable of using tannins as a carbon source.^[34]

Multidrug therapy has become of paramount importance in the fight against multidrug resistant microbial strains^[35] and such synergies will probably play an important role in the coming years. Extracts contain complex mixtures of components and thus have multiple antimicrobial properties, direct but also indirect, for example, by restoring the efficacy of antibiotics on multi-resistant strains^[17] or by modulating bacterial communication mechanisms and biofilm formation.^[36] The combination of the MG extract with antibiotics restored or enhanced the activities of streptomycin and cefotaxime by factors 4–32 (Table 3). The obtained data are in line with growing evidence that phenolic compounds (notably flavonoids) act synergistically with various antibiotics against multidrug-resistant microorganisms. For example, *in vitro*, two flavonols (kaempferol and quercetin) synergized with rifampicin against MRSA clinical isolates; combining these flavonols with rifampicin significantly increased their β -lactamase inhibitory effect.^[37] The hydroxylation and glycosylation patterns strongly influence this activity.^[38] On the other hand, lipophilicity is a common feature of several active compounds; this quality is important for their solubilization in bacterial membranes. The amphipathic kaempferol glycoside modulated the activities of the drugs by reducing the concentrations needed to inhibit the growth of bacteria.^[33]

Table 3. The effect of combination between *M. graeca* extract (MG) and antibiotics on the minimal inhibitory concentrations MICs ($\mu\text{g/mL}$).

Sample	<i>S. aureus</i> C 100459	<i>P. aeruginosa</i> ATCC 9027
MIC MG extract with cefotaxime	250	>1000
MIC MG extract with streptomycin	125	250
MIC cefotaxime with MG extract	8	32
MIC streptomycin with MG extract	4	8

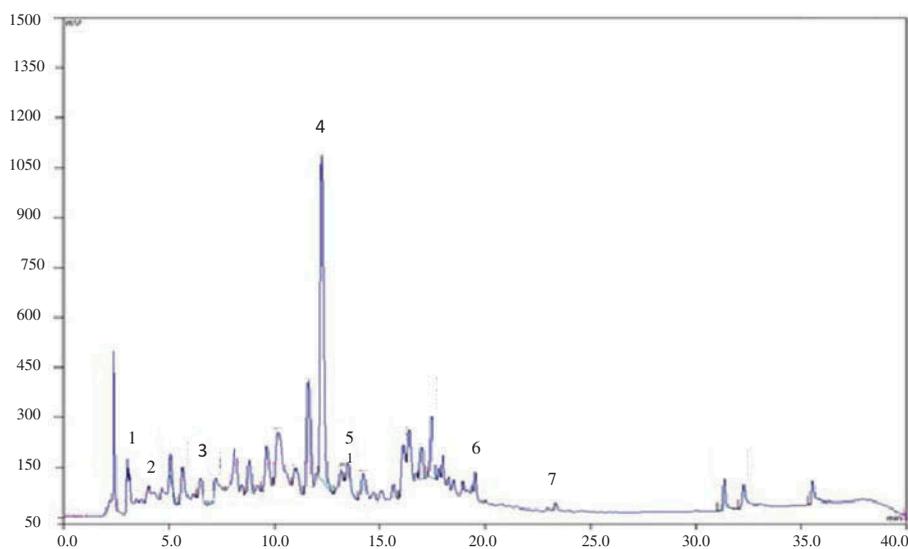


Figure 2. High-performance liquid chromatography profile of *M. graeca* extract. (1) gallic acid; (2) caffeic acid; (3) chlorogenic acid; (4) rosmarinic acid; (5) diosmin; (6) apigenin-7-glucoside; (7) apigenin. Detection at 280 nm.

Identification of polyphenolic compounds

TLC screening of the MG crude extract before and after acid hydrolysis indicated the presence of phenolic acids and flavonoids. Four phenolic acids were identified as rosmarinic, caffeic, chlorogenic, and gallic acids, verified by their intense blue fluorescent bands in UV light at 365 nm (Rf values 0.77, 0.80, 0.30, and 0.70, respectively). The presence of the phenolic acids was confirmed by HPLC-UV, with rosmarinic acid as the most abundant compound (Fig. 2). The phenolic acids such as gallic, chlorogenic, vanilic, hydroxy benzoic, sinapic, ferulic, *p*-coumaric, cinnamic, and ellagic acids were revealed previously in *Micromeria* species.^[1,9,13,19,39]

After derivatization, the flavonoids were detected on TLC as orange-yellow zones with distinct orange fluorescence. The flavonoids detected in the raw ethanolic MG extract do not match the standards used; after hydrolysis, two compounds were identified as apigenin and diosmin (Rf values 0.88 and 0.43, respectively). By comparing retention times and UV spectral characteristics with reference compounds, the flavonoids revealed by HPLC analysis in the MG extract were diosmin, apigenin-7-glucoside, and apigenin (Table 4). Tomas-Barberan et al.^[13] also detected in MG flavonoid glycosides as major constituents (luteolin and apigenin glycosides). A literature survey revealed that *Micromeria* sp. flavonoids are derivatives of acacetin, apigenin, and luteolin.^[1,13,19] Furthermore, previously, *Micromeria* species were reported to contain various flavonoids: isosakuranetin 7-rutinoside, naringenin,^[40] 5,6,4'-trihydroxy-7,3'-dimethoxyflavone, thymusin, thymonin, genkwanin, 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone, ladanein, 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone, 5-demethylnobiletin, 5-hydroxy-6,7,3',4'-tetramethoxyflavone, chrysoeriol,^[41] 5,6,4'-trihydroxy-7,3'-dimethoxyflavone, 5,6-dihydroxy-7,3',4'-trimethoxyflavone,^[42] myrcetin, hesperetin, and quercetin.^[9]

These metabolites were shown to be responsible for several pharmacological activities, notably antioxidant, antimicrobial, and enzyme inhibitory.^[43] The most abundant compounds were characterized as rosmarinic acid, diosmin, and apigenin-7-glucoside. A broad spectrum of significant pharmacological activities have been reported for these bioactive compounds.^[15,44] Accordingly the above-mentioned phenolics could be responsible for the antioxidant, antibacterial, and enzyme inhibitory effects we measured in our ethanolic extract.

Table 4. Phenolic compounds identified by HPLC in *M. graeca* extract.

Sample	Identified compounds ^a											
	Phenolic acids					Flavonoids						
	Galic acid (3.18) ^b	Caffeic acid (4.44)	Chlorogenic acid (6.65)	Ellagic acid (10.43)	Ferulic acid (10.77)	Rosmarinic acid (12.21)	Epicatechin (4.48)	Naringenin (11.31)	Diosmin (11.70)	Apigenin-7- glucoside (13.20)	Quercetin (19.59)	Apigenin (21.86)
MG	+	+	+	nd	nd	+++	nd	nd	++	++	nd	+

^aThe symbols +, ++, and +++ correspond to peak importance (from data at 280 nm).^bRetention time (min).

nd: not detected.

Conclusion

This study was conducted to investigate the antioxidant and antibacterial properties of a *M. graeca* L.) Benth. ex Rchb. ethanolic extract. With a significant phenolic content, the MG polar extract showed important scavenging properties towards ABTS^{•+}, DPPH[•], and electrochemically generated O₂^{•-}, translating into inhibition of lipid peroxidation and tyrosinase. Although inactive towards all tested bacteria (MICs > 2000 µg/mL), the MG polar extract restored the activities of cefotaxime and streptomycin on resistant *S. aureus* and *P. aeruginosa*. This promising property should be investigated in more details. The observed activities probably reflect the qualitative profile of the phenolic compounds present in this species; effectively, seven compounds (flavonoids and phenolic acids) were identified from MG, from which rosmarinic acid and diosmin were predominant.

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