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CHEMICAL COMPOSITION, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF THE ETHANOLIC EXTRACT OF *MICROMERIA FRUTICOSA* GROWING IN LEBANON

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ABSTRACT

The total phenolic and flavonoidal content of the ethanolic extract of *Micromeria fruticosa* was determined using the Folin-Ciocalteu (FC) and aluminum chloride spectrophotometric methods. Quantitative High Performance Liquid Chromatography (HPLC) was used for the identification and quantification of each phenolic compound present in the extract. Chlorgenic acid, Naringenin, quercetin and ellagic acid were found to be the major phenolic compounds of the extract. The antioxidant activity of the extract was tested using the DPPH scavenging assay. The extract has shown a strong scavenging activity with an IC_{50} = 50 µg/mL compared to the synthetic antioxidant Butylated HydroxyToluene (BHT) (IC_{50} = 91.40 µg/mL).

The antimicrobial activity was carried out using screening test and broth micro dilution method against *Staphylococcus aureas* (ATCC 6538), *Esherichia coli* (ATCC 8739) *Pseudomonas aureginosa* (ATCC 9027 *Candida albicans* (ATCC 10231) and pathogenic *Aspirigillus niger*. The extract has shown a significant antimicrobial activity against all of the tested micro organisms with the strongest activity against *Staphylococcus aureus* that was close to that of imipenem and much more than that of ampicillin.

Key words: *Micromeria fruticosa*, Antioxidant activity, Antimicrobial activity, ethanolic extract, HPLC.

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INTRODUCTION

There is an increased interest in plants' secondary metabolites like polyphenols for their therapeutic effects. Polyphenols or phenolic compounds form a large group of secondary compounds including flavonoids and phenolic acids. Both phenolic acids and flavonoids have been shown to exhibit a wide range of biological effects like antibacterial[1], anti-inflammatory[2], anti-allergic[3] and antithrombotic activities in addition to their prevention of cardiovascular diseases[4]. This can be associated to their strong antioxidant activity as these compounds are capable of blocking the effect of the reactive oxygen species in different aspects like trapping these reactive oxygen species, reducing the alkoxyl and peroxyl radicals, chelating transition metals and obstructing enzymes involved in the generation of superoxide anions[5]. Consequently, more focus is done on plant derived antioxidants, studies have shown that the dietary intake of these plants can reduce the level of reactive oxygen species in the body, and thus lower the risk of degenerative diseases, without exhibiting any considerable side effects like the synthetic antioxidants BHT, which were associated to carcinogenesis[6].

Chromatographic techniques are widely used for the separation and identification of the plant derived phenolic acids and flavonoids. Since GC/MS in this case requires derivatization, which is time consuming and requires extreme conditions, all focus is done on High Performance Liquid Chromatography (HPLC) as the extract is directly loaded on the machine without prior treatment. By using HPLC, the herbal content will be identified and quantified by comparing the retention times of the peaks obtained to those of external standards, which are run on the machine under the same conditions and at different concentrations[7].

In a study conducted by WHO in 2000[8], about 80% of the world population still counts on herbal medicine although the active compounds of these plants are mostly unknown. As a result, a lot of the ongoing researches are focusing on these herbs in search for new drugs and to find a scientific explanation to the usage of these herbs in traditional medicine[9]. One of these herbs is *Micromeria fruticosa* (*Lamiaceae*), which is an aromatic plant that grows in the Mediterranean region like Lebanon, Syria and Turkey. In herbal medicine, this plant is widely used to treat various diseases including abdominal pains, diarrhea, colds, wounds and skin infections[10].

In this paper, we screened the extracts of these *Lamiaceae* herbs for their antimicrobial and antioxidant activity and identify its phenolic content using HPLC. That should complement to their previously known therapeutic value and improve the popularization of those somehow overlooked common herb species.

EXPERIMENTAL

Materials and methods

Plant material

The aerial parts of the two samples of *Micromeria fruticosa* were collected from the Lebanese Bekaa valley at 1300 m above sea level and authenticated by Dr. Ali Chakas, Botanist, Lebanese University, Faculty of Science III, one at the full flowering stage in July and the other in a later stage in October. The samples were dried at room temperature.

Solvents and reagents

The solvent used for extraction of *Micromeria fruticosa* was analytical grade ethanol; while those used for the HPLC analysis were HPLC grade acetonitrile and Milli-Q (Millipore Australia Pty. Ltd.) distilled water.

Aluminum chloride, potassium acetate, FC reagent, Na_2CO_3, DPPH and the standards Gallic acid, chlorogenic acid, syringic acid, vanillic acid, cafeic acid, hydroxybenzoic acid, sinapic acid, ferulic acid, p-coumaric acid, cinammic acid, myrcetin, hesperetin, quercetin, naringenin, chrysin and ellagic acid were all purchased from Sigma Aldrich (Steinheim, Germany).

Standards

All standards were prepared as stock in acetonitrile. Working standards were made by diluting stock solutions in acetonitrile to yield concentrations ranging between 25-125 mg/L.

Sample preparation

The collected sample of *Micomeria fruticosa* was percolated with ethanol (70%) at room temperature for two days, filtered, then solvent dried by vacuum evaporation. The extract was kept at 20°C until usage. For HPLC analysis, 10.5 mg of the extract was dissolved in 1 mL of acetonitrile and directly injected to the HPLC.

Determination of total flavonoids

The aluminum chloride spectrophotometric method was used for the quantification of the total phenolic content of the extract as described by Hossian and Rahman[11] with slight modifications. 0.5 mL of ethanolic extract (9.67 mg/mL), 0.1 mL of aluminum chloride (10%), 0.1 mL of potassium acetate (1 M) and 4.3 mL of distilled water were mixed together

then incubated for 30 minutes. The absorbance was measured at 415 nm and Quercetin was used to make the calibration curve. Results were expressed in mg of quercetin/g dry sample.

Determination of total phenolics

Folin-Ciocalteu (FC) spectrophotometric method was used for the quantification of the total flavonoidal content of the extract based on procedure described by Barros et al.[12] with slight modifications. 1 mL of the ethanolic extract (4.835 mg/mL) was mixed with 0.5 mL of Folin-Ciocalteu reagent (1 N) and diluted by 5 mL of distilled water. After 5 minutes, 350 µL of Na_2CO_3 15% was added. The tubes were allowed to stand for 1.5 hr in dark for color development. Absorbance was measured at 725 nm and Gallic acid was used to make the calibration curve. Results were expressed in mg of gallic acid/g dry sample.

Antioxidant activity of the ethanolic extract

The radical scavenging activity of the BHT and a prepared solution of the ethanolic extract (S_0 = 9.67 mg/mL) were tested. DPPH radical (1.75 mM) was mixed with a range of (20-60 µL) of S_0 and the total volume was adjusted to 4 mL by ethanol leading to a range of 0.048-0.145 mg/mL solutions of ethanolic extract. The reaction mixture was shaken and then incubated at room temperature in dark for 45 minutes. The DPPH radical inhibition was measured at 517 nm by using a Shimadzu UV spectrophotometer. Using the same conditions, BHT was used as a reference to compare its results to those of the ethanolic extract.

Antimicrobial activity of the extract

The ethanolic extract of *Micromeria fruticosa* was tested against one Gram positive bacteria *Staphylococcus aureas* (*S. aureas*) (ATCC 6538), two Gram -ve bacteria, *Esherichia coli* (*E. coli*) (ATCC 8739) and *Pseudomonas aureginosa* (*P. aureginosa*) (ATCC 9027), and two fungi, *Candida albicans* (ATCC 10231) and pathogenic *Aspirigillus niger* (*A. niger*). The screening zone inhibition zone diameter (IZD) was used to determine the antimicrobial activity of the extract. The used organisms were standard strains provided by the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Beirut Arab University. A colony of each of the microorganisims was separately cultured in Mueller Hinton broth for 24 hrs. 1 mL of this suspension is then mixed with 100 mL of sterile molten nutrient agar (oxoid) that was maintained at 45°C. Each of the inoculated media was mixed well and poured into sterile 10 cm diameter petri-dishes. After setting, a well (5 mm) was made by a sterile cork borer in the agar medium for each microorganism and 300 µL of the ethanolic extract were taken by a micropipette and added to the wells. The plates were incubated at 37°C for 24 hrs for bacteria and at 34°C for 72 hrs for fungi. Then the diameter of the inhibition zones were measured and expressed in milliliters. A broth micro dilution

method was used to determine MIC. All tests were performed in nutrient agar broth. Serial dilutions of the extract were prepared in a series of 5 tubes ranged from 3 to 3/16 (g/mL). The final concentration of each strain was adjusted to 10^6 CFU/mL. Plates were incubated at 37 degrees for 24 hrs after which the Minimum Inhibitory concentration (MIC) was recorded as the lowest concentration of essential oil at which the microbes did not show any visible growth. Each test was performed in three replicates and repeated twice. Ampicillin and Imepenem (broad spectrum antibiotics) served as positive control.

HPLC Analysis

A simple and quick reversed phase method for determination of phenolic acids and flavonoids was developed. Chromatography analysis was performed with the use of liquid chromatographic system, which consisted of Prominence Liquid Chromatographic Shimadzu instrument with UV- Detector-SPD-20 A. The separation was carried out on Ascentis RP-Amide (15 cm x 4.6 mm ID, 5 µm particles) reversed phase column. Column temperature was maintained at 25°C. The mobile phase was a gradient elution of water containing 0.085% orthophosphoric acid (solvent A) and acetonitrile (solvent B) at a flow rate 1 mL/min. The gradient program of solvent A in B (v/v) was as follows: 0-30 min 85% A; 30-35 min 65% A; 35-60 min 15% A. the injection volume for all samples was 20 µL. For detection, chromatograms were monitored at 280 nm. Identification of phenolic acids and flavonoids was based on retention times in comparison with standards. The quantification was carried out using the external standard method. Stock solution of standard compounds at concentration 1 mg/mL each was prepared in acetonitrile, and several dilutions with acetonitrile were made. The solution of standards at various concentrations (25-125 mg/L) was injected into the HPLC system and the calibration curves were established for each standard compound. The concentration of the compound was calculated from peak area according to calibration curves. The amount of each phenolic acid and flavonoid was expressed as milligram per gram dry sample.

RESULTS AND DISCUSSION

Total phenolic and total flavonoid content

The calibration curves of Gallic acid and Quercetin showed linearity of the method over the concentration range analyzed with values of correlation coefficient 'R' of 0.999 and 0.9969, respectively (Fig. 1). Using the calibration curves, the total phenolic content of *Micromeria fruticosa* is 39.87 mg gallic acid/g extract and its total flavonoidal content was 36.28 mg quercetin/g extract (Table 1). The results show that *M. fruticosa* contains significant amounts of phenolic compounds which are mostly flavonoids.

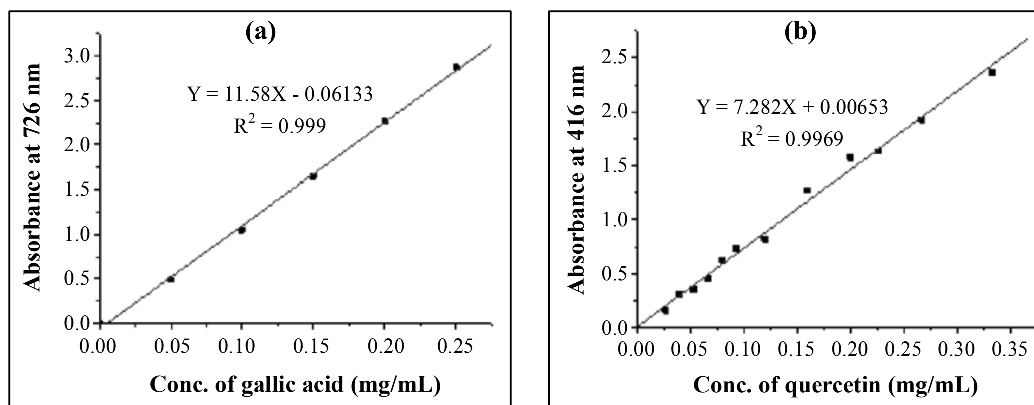

Fig. 1: Calibration curves of Gallic acid (a) and Quercetin (b)

Table 1: Total phenolic and total flavonoidal content of the ethanolic extract

	Phenolic content	Flavonoidal content
Standard curve	Gallic acid	Quercetin
Absorbance	725 nm	415 nm
Content/g extract	39.87 mg/g	36.28 mg/g
R^2 value	0.999	0.9969

HPLC Analysis

After extraction, the content of phenolic substances was determined by HPLC quantitative analysis. The HPLC chromatogram of *Micromeria fruticosa* is presented in Figure 2. The amounts of identified phenolic compounds detected in the extract are represented in Table 2.

Table 2: Chemical composition of the ethanolic extract using HPLC

Compound	mg/g of extract	Retention time (min)
Gallic acid	0.067	1.828
Chlorogenic acid	1.123	2.224
Vanilic acid	0.065	9.157
Hydroxybenzoic acid	0.169	12.339

Cont…

Compound	mg/g of extract	Retention time (min)
Sinapic acid	0.059	14.147
Ferulic acid	0.05	16.778
p-coumaric acid	0.031	17.521
Cinnamic acid	0.001	30.516
Myrcetin	0.196	33.828
Hesperetin	0.962	36.254
Naringenin	0.868	36.775
Quercetin	0.884	37.147
Ellagic acid	0.693	40.142

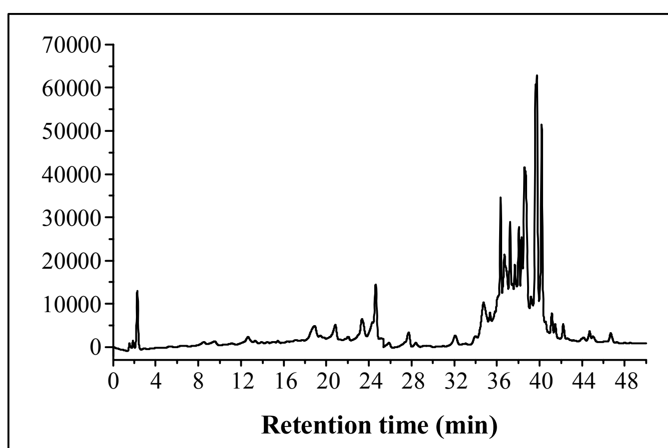

Fig. 2: The HPLC chromatogram of *Micromeria fruticosa* extract

As indicated by Table 2, the major phenolic acids are chlorogenic acid and ellagic acid whereas the major flavonoids are hesperetin, naringen and quercetin. This is in accordance with the results obtained by the FC and aluminum chloride photometric assays that have shown that most of the phenolic compounds in the extract belong to the family of flavonoids. Moreover, the presence of these phenolic compounds in this extracts reveals a possible medical importance of the extract. Naringenin has an anti-inflammatory effect and is considered an immune system modulator as it reduces hepatitis C virus production by infected hepatocytes (liver cells) in cell culture[13]. Hesperetin suppresses tumor cell proliferation and bioactive hormone production making this compound a potential candidate for treatment of carcinoid cancer[14]. Ellagic acid has antiproliferative properties due to its

ability to directly inhibit the DNA binding of certain carcinogens, including nitrosamines[15,16] and polycuclic aromatic compounds[17] ellagic acid has a chemoprotective effect in cellular models by reducing oxidative stress[18].

Antioxidant capacity

The degree of inhibition was calculated using the following equation:

$$\% \text{ DPPH inhibition} = (A_{Blank} - A_{Sample}) / A_{Blank} \times 100$$

The results are indicated in Figure 3 and Table 3.

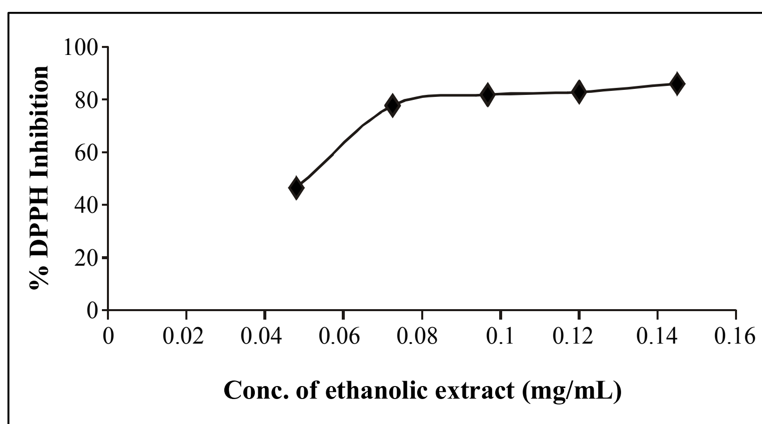

Fig. 3: % of DPPH inhibition of the ethanolic extract

Table 3: Radical scavenging activity of ethanolic extract and BHT

	IC_{50} µg/mL
Ethanolic extract	50
BHT	91.40

The ethanolic extract of the *Micromeria fruticosa* inhibited the activity of DPPH radical in a dose response relationship. In this case, the IC_{50} of the extract was 50 µg/mL, which was significantly less than that of BHT (91.40 µg/mL).

Antimicrobial activity

The results showed that the phenolic components of the extract of *M. fruticosa* have an important antibacterial activity against *Staphylococcus aureas*. In addition, a significant

antifungal activity against *Candida albicans* was also evident (Table 4). These results were even more important than those of the commonly used antibiotics like Imipenim and ampicillin (Table 5). The results of MIC indicated *S. aureus* had the lowest MICs (3/8), the highest MIC was for *C. albicans*.

Table 4: Antimicrobial activity of plant expressed as inhibition zone diameter

Tested material		IZD of Tested samples (mm)				
		Gram-positive bacteria	Gram-negative bacteria		Fungi	
Sample	**Sample**	***S. aureus***	***E coli***	***P. aeruginosa***	***C. albicans***	***A. niger***
1	**Extract of *M. fruticosa***	30	0	0	15	0
2	**Pure ethanol**	12	10	11	0	0

IZD: Inhibition zone diameter

Table 5: Results of antimicrobial activity of the reference antibiotics expressed as Inhibition Zone Diameter (IZD) in mm of the microorganisms

Test material		Tested microorganism			
Antibiotic	**Disc dimension**	Gram-positive bacteria	Gram-negative bacteria		Fungi
		S. aureus	***E. coli***	***P. aeruginosa***	***C. albicans***
Ampicillin	5 mm	10	23	25.6	11
Imipenem	5 mm	28	31	23	20

Table 6: Determination of minimum inhibitory concentration (MIC) of ethanolic extract of *Micromeria fruticosa*

Concentration of the extract g/mL	3	1.5	0.75	0.375	0.1875
Growth of *S. aureus*	-	-	-	-	+
Growth of *E. coli*	-	-	-	+	+
Growth of *P. aeruginosa*	-	-	+	+	+
Growth of *C. albicans*	-	+	+	+	+

The chemical composition of the ethanolic extract of *Micromeria fruticosa* was determined using quantitative HPLC and other spectrophotometric methods revealing the presence of significant amounts of phenolic compounds, mainly flavonoids, of important medicinal impact in the extract. The DPPH radical scavenging activity of the extract has shown a remarkably better antioxidant activity of the extract than the commonly used antioxidant BHT. This can be justified by the presence of phenolic acids and flavonoids in the extract as it was shown by the HPLC analysis results (Table 2) as the antioxidant activity of some plants is mainly attributed to its Phenolic compounds that act as reducing agents, hydrogen donators and singlet oxygen quenchers[19,20].

The extract has shown an advanced antimicrobial activity against *Staphylococcus aureas* and *Candida albicans*c compared to the commonly used antibiotics. These results support partly the usage of *Micromeria fruticosa* as traditional remedies for some infections and suggest that it can be a new medicinal resource for multi-resistant microbes specially that *Staphylococcus aureas and Candida albicans* are considered to be major nosocomial pathogens responsible for a wide variety of infections.

This study supports the usage of this herb in herbal medicine, and suggests that more work should be done on this herb as it contains several phenolic compounds of various medicinal.

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