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Antioxidant Activity of Mint (*Mentha piperita* L.) of Greek Flora and Identification of its Bioactive Compounds.

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Abstract

Mentha spieces are used in medicine worldwide. In this study, *Mentha piperita* L. was examined. The aim of this work was the determination of the total phenolic content in the plant extract by Folin-Ciocalteu method and the estimation of the antioxidant activity of the mint extract using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical. Qualitative and quantitative determination of the bioactive compounds included in the plant extract was performed by using High Performance Liquid Chromatography coupled with diode array detection. Another analytical method, mass spectrometry analysis was used to identify the m/z ratio of the secondary metabolites in the sample. Among all the identified phenolic compounds, the most abundant compound was caffeic acid from phenolic acids and eriodictyol from flavonoids.

Keywords: *Mentha piperita* L.; Phenolic compounds; HPLC-DAD analysis; MS analysis; Antioxidant activity

Introduction

Spices and herbs are known to serve as powerful antioxidants. Antioxidants are the substances which significantly destroy the free radicals—reactive oxygen species responsible for degenerative diseases. In recent years, natural phytochemicals existing in herbs and spices have been widely used to cure, inhibit or reduce the risk of human diseases [1,2]. Phytochemicals have potential health benefits due to their antioxidant activities and inhibitive effects against oxidative damage that has been implicated in a number of illnesses, specifically cancer and cardiovascular diseases [3]. Among the phytochemical substances, phenolic compounds, including phenolic acids and flavonoids, are the major groups of natural components in plants. These bioactive compounds vary in type, number and position of functional group [4].

Mint (*Mentha*) belongs to the Lamiaceae family and is considered a folk medicine for heavy colds and the digestive system [5]. It contains about 25 different species such as, *Mentha piperita*, *Mentha spicata*, *Mentha arvensis*, *Mentha* aquatic and others. According to the literature, *mentha* spp. have been utilized in many therapeutical and pharmaceutical applications and have been used as antimicrobial, anticancer, antispasmodic, anti-inflammatory, antidiabetic drugs [6]. Except for medicinal purposes, mint is used in many cosmetic and flavoring industries [7]. In this study, *Mentha piperita* L. is examined for the identification of its secondary metabolites and its antioxidant activity. We evaluated the presence of phenolic compounds in mint extract with analytical methods, HPLC-DAD and MS analysis. In addition, our study was performed to evaluate the antioxidant capacity of the aromatic plant and its total phenolic content with Folin-Ciocalteu method.

Materials and Methods

Reagents and standards

2,2-Diphenyl-1-picrylhydrazyl (DPPH•), Folin-Ciocalteu phenol reagent, sodium carbonate, formic acid and standards compounds such as naringenin, thymol, carvacrol, luteolin, quercetin, kaempferol, apigenin, apigenin-7-glucoside, rutin, eriodictyol, vanillic acid, rosmarinic acid, p-coumaric acid, hydroxybenzoic acid, ferulic acid, caffeic acid, chlorogenic acid, benzoic acid, gallic acid were purchased from DR EHRENSTORFER GmbH. Methanol, acetonitrile and ultra-pure water were of HPLC grade and ethanol

was of analytical grade. All solvents were purchased from Merck (Darmstadt, Germany).

Extraction and preparation of natural extract

Dried leaves of mint (*Mentha* L.) were collected from Northern Greece and specifically from Serres. The leaves were cut in small pieces and then they were ground to a fine powder using a mechanical blender. The dried powdered leaves were extracted by maceration with 10% ethanol and 90% distilled water at room temperature for 14 days with occasional shaking [8]. After filtration, 50ml of liquid extract were remained and centrifuged at 8000rpm for 15minutes and the supernatant was collected. The solvent was removed using a rotary evaporator at 50˚C. The extract was dissolved in methyl alcohol and was stored at 4˚C for further use. The sample was filtered through a 0.22μm PVDF filter before the injection in the HPLC system.

Analytical procedures

High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) analysis

The HPLC-DAD analysis proposed by Kouri et al. (2007) [9] with some modifications was used in order to identify the bioactive compounds into the natural extracts. The HPLC apparatus is a Hitachi LaChrom Elite HPLC system and consisted of an autosampler, a gradient pump, a column oven and a diode array detector. A SVEA C18 column, 150 mm x 4.6, 5μm particle size from Nanologica, maintained at 30˚C was used with a flow rate of 0.5mL min^{-1}. The solvent system consisted of (A) water, (B) methanol and (C) acetonitrile, each containing 1% formic acid. Solvent gradient was performed as follows: initial 90% A, 6% B, 4% C 0-5 min, 85% A, 9% B, 6% C 5-30 min, 0% A, 85% B, 15% C 30-60 min, 90% A, 6% B, 4% C 60-63min. The total time of analysis was 65 min. The injected volume of sample and standards was 20μl. The spectra were required in the range 200-400nm and the chromatograms are represented at 225, 280, 355 and 370nm. All the analyses were made in triplicate.

Mass Spectrometry (MS) Analysis

MS analyses were performed on a Mass Spectrometer Advion equipment. The mass analyzer is a single quadrupole. The quadrupole is consisted by four parallel rods that use electric fields to filter ions based on the m/z ratio. The MS system was operated in Atmospheric Pressure Chemical Ionization (APCI) positive mode and the conditions used were Capillary Temperature 200˚C, Capillary Voltage 180V, Source Voltage Offset 25V, Source Voltage Span 20V, Source Gas Temperature 35˚C and APCI Corona Discharge 5μA. For full scan MS analysis, the spectra were recorded in the range 100-800m/z.

Determination of antioxidant capacity

Free radical scavenging activity by the use of a stable DPPH• radical. The DPPH• radical-scavenging activity was determined using the method proposed by Dudonné et al. (2009) [10] with some modifications. The DPPH• is a blue-colored stable free radical, which is reduced to 2,2-diphenyl-1-picrylhydrazine (pale yellow), by reacting with an antioxidant [11]. The DPPH• solution of $6x10^{-5}$ M was prepared in methanol. 3.4mL of this solution was subsequently added to 100μl of various concentrations of the mint extract (A_{Sample}). The samples were remained in dark for 45minutes and allowed to react at room temperature. The decrease in absorbance was measured at 517nm. The absorbance of the DPPH• radical without extract was also measured ($A_{control}$). Total antioxidant activity is expressed in μg/ml gallic acid. The results are expressed as the amount of antioxidant needed to cause a 50% reduction in DPPH absorption (IC50). The % inhibition is calculated by the following formula:

$$\%inhibition = \frac{A_{control} - A_{sample}}{A_{control}} x100 \quad (1)$$

All determinations were performed in triplicate.

Determination of total phenolic content

The total phenolic concentration in aqueous natural extract was determined by the Folin-Ciocalteu method using gallic acid as the standard [12]. Briefly, 200 μl of aqueous solutions of gallic acid were mixed with 0,8 ml Na2CO3 (7,5 % in deionized water) and 1 ml Folin-Ciocalteu reagent (diluted 1:10). The concentration range of gallic acid is from 200μg/ml to 12.5 μg/ml. The natural extracts were mixed with the same reagents as described above. The mixtures were mixed with vortex and incubation was done for 90 minutes at room temperature at darkness. The absorbance was read at 765 nm using spectrophotometer <<Thermo Spectronic>> [13]. All measurements were carried out in triplicate. The results are expressed as mg GAE/g of dry extract.

Results and Discussion

Analytical procedures

High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) analysis

The qualitative identification of bioactive compounds in natural extracts is depended on their retention time and their maxima wavelengths. The concentration of phenolic compounds in mint extract was calculated based on the calibration curve of standards [14]. All standards solutions are dissolved in methanol. A detailed table with all examined standard compounds is shown below (Table 1). The spectra of the mint extract is represented at 280nm. The majority of the components show a maximum absorption and have a successful separation at this wavelength (Figure 1). The quantity of the studied substances in the sample is shown in Table 1. The most abundant phenolic acids identified in mint extract were caffeic acid (116.89±0.28 ppm) and benzoic acid (41.92±0.61ppm) and the main flavonoid is eriodictyol (32.23±0.75 ppm). Apigenin was found in traces in the samples. The peaks of the chromatogram can confirm these results.

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Table 1: Standard compounds their retention time. calibration curves. maxima wavelengths in ultraviolet region and their concentration in mint extract.

	Standard Compound	Retention Time (min)	Calibration Curve	Regression Coefficient (R2)	Maxima Wavelengths (nm)	Concentration in the Sample (ppm)
A	Naringenin	50.11	y=392749x+600702	0.998	235,289	-
B	Thymol	57.99	y=140232x+563914	0.9953	215,276,345	-
C	Carvacrol	57.65	y=96580x+159301	0.9987	221,275	-
D	Luteolin	49.80	y=201134x+343231	0.9961	256,263,349	27.64±0.32
E	Quercetin	49.24	y=208457x+441912	0.9992	230,256,370	6.25±0.46
F	Kaempferol	52.03	y=265195x-28475	0.998	250,315	-
G	Apigenin	52.21	y=407824x+599220	0.9992	267,338	0.10±0.03
H	Apigenin-7-glucoside	43.20	y=475911x-3E+06x	0.9918	206,267,337	-
I	Rutin	40.63	y=101038x+244152	0.9948	258,356	8.32±0.59
J	Eriodictyol	46.96	y=462783x+901504	0.9993	205,230,288	32.23±0.75
K	Vanillic acid	22.55	y=280308x+1E+06	0.9972	208,265,292	-
L	Rosmarinic acid	44.50	y=342476x-1E+06	0.9948	206,245,330	6.03±0.52
M	p-coumaric acid	33.50	y=1E+06x-268063	0.9962	216,309,395	32.21±1.02
N	Ferulic acid	36.77	y=361100x+667162	0.9984	210,322	31.92±0.83
O	Caffeic acid	24.57	y=460642x-243764	0.9934	209,240,323	116.89±0.28
P	Hydroxybenzoic acid	19.29	y=216500x-261891	0.9962	210,260,388	30.86±0.92
Q	Benzoic acid	42.23	y=56565x+111379	0.9969	273,370	41.92±0.61

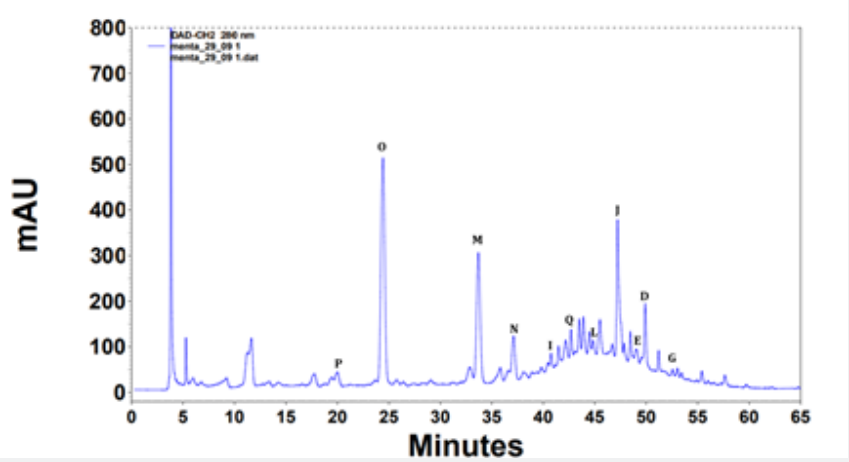

Figure 1: Chromatogram of mint extract on HPLC-DAD apparatus.

Mass spectrometry (MS) analysis

The constituents that were identified in mint extract were confirmed by MS spectra. The main ions, which are characteristic for each compound, are shown in Table 2. Rutine could not be identified with MS analysis. The fragmentations of the compounds were not observed at low capillary voltages. After adjusting the capillary voltage at 180 V, molecular weights and main ions of standards were identified in the studied extract.

Determination of antioxidant activity

From the equation y= 23.761ln(x)+3.897 from the standard reference curve of gallic acid (Figure 2a) turns out that IC50= 6.96±0.25μg/ml. The concentration range of gallic acid is from 3.43μg/ml to 55μg/ml. In the same way, it was calculated IC50 of mint extract (IC50=7.34±0.54μg/ml) (Figure 2b). The identification of IC50 is the most suitable way to correlate the antioxidant activity of numerous natural extracts. The lowest value corresponds to the best antioxidant activity of the sample.

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In addition, the absorbance of different concentrations of mint sample at different times (0min, 45min and 24h) was measured and percentage inhibition was calculated (Table 3) [15]. The same procedure was repeated for the standard Gallic acid (Table 4).

Table 2: m/z ratio of standard compounds.

Standards	Main ions observed (m/z)
Luteolin	287.2
Quercetin	303.2
Apigenin	271.2
Eriodictyol	289.1
Rosmarinic acid	361.3
p-coumaric acid	165.2
Ferulic acid	177.1
Caffeic acid	135.0
Hydroxybenzoic acid	139.1
Benzoic acid	123.1

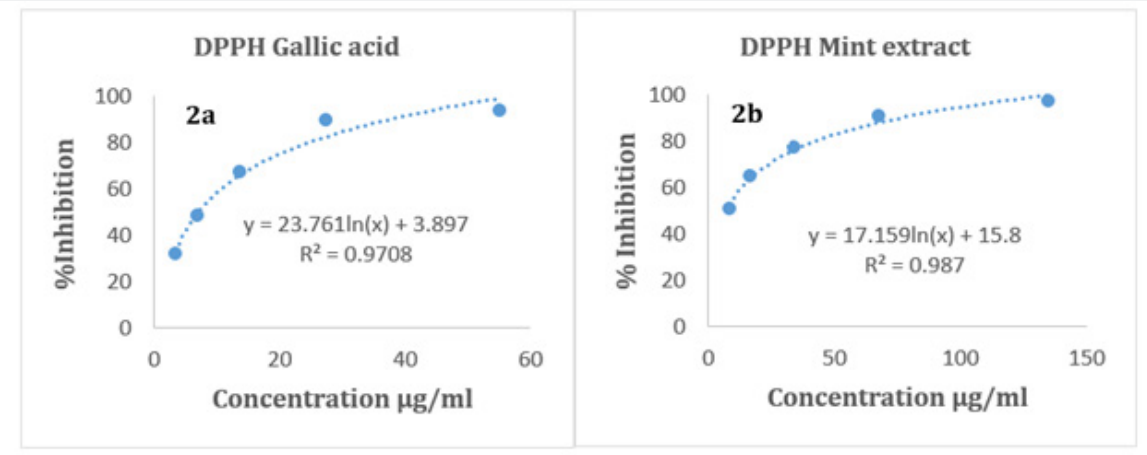

Figure 2: DPPH radical scavenging activity of a) standard gallic acid and b) mint extract.

Table 3: Absorbance and percentage inhibition of different concentrations of mint extract at three different times.

Concentration of sample (µg/ml)	Absorbance			%Inhibition		
	0 min	45 min	24 h	0 min	45 min	24 h
135	0.023	0.023	0.020	97.29	97.29	97.6
67.5	0.079	0.079	0.059	90.70	90.70	92.9
33.75	0.193	0.193	0.162	77.29	77.29	80.60
16.88	0.298	0.298	0.273	64.94	64.94	67.3
8.44	0.419	0.419	0.382	50.70	50.70	54.25
4.22	0.664	0.664	0.632	21.88	21.88	24.31
Radical DPPH	0.850	0.850	0.835			

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Table 4: Absorbance and percentage inhibition of different concentrations of Gallic acid at three different times.

Concentration of sample (μg/ml)	Absorbance			%Inhibition		
	0 min	45 min	24 h	0 min	45 min	24 h
55	0.053	0.053	0.042	93.76	93.76	94.97
27.5	0.088	0.088	0.075	89.65	89.65	92.22
13.8	0.28	0.28	0.263	67.00	67.00	68.5
6.88	0.438	0.438	0.413	48.47	48.47	50.54
3.44	0.578	0.578	0.556	32.00	32.00	33.41
1.72	0.734	0.734	0.703	13.65	13.65	15.81
Radical DPPH	0.850	0.850	0.835			

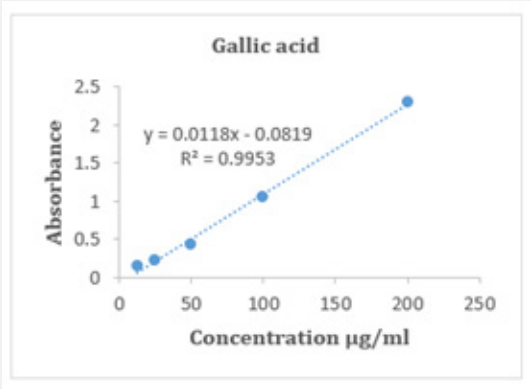

Figure 3: Standard reference curve of Gallic acid.

It is obviously noticed that the absorbance of the freshly prepared sample was the same with this, which was calculated after 45min. This also applies to the absorption of the DPPH radical. After one day, a small decrease in the absorption values was observed. It was observed that the mint extract had IC50=9.20±0.13μg/ml after 24hours, while IC50 of Gallic acid was similar with the other times (0min, 45min). Extract of *Mentha piperita* L. shows lower antioxidant capacity during a day.

Total phenolic content

Folin Ciocalteu method was used to estimate the phenolic compounds in the sample. The standard curve of gallic acid is represented in Figure 3. The absorbance of the sample at 765nm is A765=0.781. From the equation y=0.0118x-0.0819 with R2=0.9953 the total phenolic content is measured. The mint extract has 541μg GAE/mg of dry extract. The antioxidant activity of *Mentha piperita* L. depends on the included large amount of phenolic compounds. According to other research, the major phenolic acids of *Mentha piperita* L. extract are gallic acid and caffeic acid, while ferulic acid and p-coumaric acid are not detected [16]. The large amount of total phenolic content (541μg GAE/mg of dry extract) mint extract can be confirmed after the comparison with other studies [16,17]. According to the survey of Brahmi et al. (2017), *Mentha piperita* L. includes a variety of phenolic acids such as caffeic, ferulic, rosmarinic, syringic, chlorogenic, vanillic acid and flavonoids such as rutin, quercetin, luteolin and their glucosides. Our results are comparable to the researches above.

Conclusion

In conclusion, mint extract and especially extract of the spice *Mentha piperita* L. is a health effective plant. At the present study, the antioxidant activity of the herb was evaluated after the identification of total phenolic content by Folin-Ciocalteu method. HPLC analytical procedure was used in order to quantify the secondary metabolites in the sample and MS analysis confirmed the presence of these compounds. The concentration range of bioactive compounds varied from 0.10ppm to 116.89ppm. The plant proved to be rich in phenolic acids, caffeic acid and benzoic acid. In a subsequent survey, the antimicrobial activity of mint extract will be studied. These investigations are essential for the expansion of use of this plant in industries.

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