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ANTIOXIDANT POTENTIAL AND POLYPHENOLIC CONTENT OF ROMANIAN *OCIMUM BASILICUM*

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This study sought to carry out a screening of the polyphenols from the aerial parts of four samples of *Ocimum basilicum*. HPLC-MS method was used to investigate the presence of phenolic compounds in the studied samples; caftaric, cafeic, chlorogenic, p-coumaric acids and rutin were identified. The total polyphenolic content was determined by using the Folin-Ciocalteu reagent, the flavonoid and the caffeic acid derivatives contents were detected using spectrophotometric methods. In addition, the antioxidant activity was evaluated by using the stable free radical diphenylpicrylhydrazyl (DPPH). The quantitative determinations made by several spectrophotometric methods showed different results, samples of culture of *Ocimum basilicum* being richer in phenolic compounds than the commercial samples. The antioxidant capacity in all cases was moderate, even if the polyphenols content was high.

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1. Introduction

Ocimum basilicum L. (*Lamiaceae*), the basil, is an aromatic herb that is widely used for medicinal porposes and to add a distinctive aroma and flavor to food. The basil contains volatile oil, caffeic acid derivatives, flavonoids responsible for the antimicrobial, diuretic and digestive stimulant properties of the drug. Preparations of basil are used for supportive therapy for feelings of fullness and flatulence, for stimulation of appetite and digestion [1]. Major aromatic compounds from volatile oils of basil have showed anti-oxidative activity. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions. Usually, the antioxidant activity can be related with the polyphenolic content of plant material. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and tripletoxygen, or decomposing peroxides. Many of these phytochemicals possess significant antioxidant capacities that may be associated with lower incidence and lower mortality rates of cancer in human populations [2,3,4,5].

In this regard, we evaluated the phenolic profile of the aerial parts of *Ocimum basilicum* (*Basilici herba*) by HPLC-MS analysis and the antioxidant activity. This study is also to complete the basis for a scientific rationale of the therapeutic use of medicinal teas of basil.

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2. Experimental

2.1 Materials and samples preparation

The aerial parts of *Ocimum basilicum* L. were harvested in July 2011 (Cluj, Romania, sample 1) and in June 2011 (Dolj, Romania, sample 2). The samples 3 and 4 were coming from Romanian commercial medicinal teas. Voucher specimen (No. 792) was deposited in the Herbarium of the Department of Pharmaceutical Botany of the Faculty of Pharmacy ("Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania).

The samples preparation: 0.5 g of the powder was extracted with 20 ml of 50% and 80% ethanol, for 30 min on a water bath, at 60°C. The samples were then cooled down and centrifuged at 4500 rpm for 15 min, and the supernatant was recovered [6,7]. For tincture (1:10 extraction), the dried and powdered plant materials (10 g) were extracted with 100 g 70% ethanol by maceration, for 10 days, at room temperature [6]. Extraction and preparation of samples for HPLC analysis: the dried and powdered plant material (5 g) was extracted with 100 mL methanol 50% at 80^{o}C for 30 minutes on a water bath and then they were sonicated for 5 minutes and finally heated again for another 10 minutes at 80^{o}C [8].

2.2 HPLC–MS analysis

Apparatus and chromatographic conditions: we used an Agilent 1100 HPLC Series system (USA) equipped with a degasser G1322A, a quaternary gradient pump G1311A and auto sampler G1313A. The separation was done using a Zorbax SB-C18 reverse-phase analytical column (100 mm x 3.0 mm i.d., 3.5 μm particles. The working temperature was 48^{0}C and the detection of the compounds was performed at 330 nm (first 17 min from chromatogram) and 370 nm (from 17 to 38 min) using a G1311A diode array detector system. The chromatographic data were processed using ChemStation and DataAnalysis software from Agilent, USA. The mass chromatographic spectrometer was an Agilent 1100 SL Series ion trap equipped with turbo-ionspray (ESI, electrospray ionisation) interface, negative ion mode. ESI settings were as follows: temperature, 360^{0}C; dry gas, nitrogen; nebuliser, nitrogen at 65 psi. The mobile phase was a binary gradient prepared from methanol and acetic acid 0.1% (v/v). The gradient begun with a linear gradient started at 5% methanol and 42% methanol over first 35 minutes, followed by isocratic elution with 42% methanol over the next 3 minutes. The flow rate was 1 mLmin^{-1}, the injection volume was 5 μl and data were collected at 330 nm.

The identification of the polyphenols in the samples was made by comparison of their retention times with those of pure standards and confirmed by HPLC-MS. Quantitative determinations were made using an external standard method. Quantification was performed on the basis of linear calibration plots of peak area against concentration. Calibration lines were constructed based on five concentration levels of standard solutions within 0.5 to 50 mgL^{-1} range. Accuracy was checked by spiking samples with a solution containing each phenolic compound in a concentration of 1 mg mL^{-1}. All compounds were identified by both standard addition and comparison of their retention times and MS spectra with those of standards in the same chromatographic conditions [8].

2.3 Determination of total phenolic compounds content

The total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteau method with some modifications [7,9,10]. Briefly, 0.5 ml of 50% and 80% ethanol extracts were mixed with 1.0 ml Folin–Ciocalteu reagent, 10.0 ml distilled water (R) and diluted to 25.0 ml with a 290 g/l sodium carbonate solution R. The samples were incubated in the dark for 30 min. The absorbance was measured at 760 nm using water as compensation liquid. Gallic acid was used as standard for the calibration curve and was plotted at 0.02, 0.04, 0.06, 0.08, and 0.10 mg/ml gallic acid that was prepared in methanol:water (50:50, v/v). TPC values were determined using an equation that was obtained from calibration curve of gallic acid graph (R^2:0.99904).

2.4 Determination of total flavonoid content

Spectrophotometric aluminum chloride method was used for the flavonoid determination [6]. Briefly, 5 mL of 50% and 80% ethanol extracts were mixed with 5.0 mL of sodium acetate 100 gL^{-1}, 3.0 mL of aluminum chloride 25 g L^{-1}, and filled up to 25 mL by methanol in a calibrated flask. The absorbance was measured at 430 nm. The total flavonoid contents values were determined using an equation that was obtained from calibration curve of rutin graph (R^2: 0.999592).

2.5 Determination of caffeic acid derivatives content

The total phenolic acids content in the plant material was determined using the spectrophotometric method with Arnow's reagent according to the procedure described in the Romanian Pharmacopoeia X [6]. Caffeic acid derivatives were determined from an aliquot of 50% and 80% ethanolic extracts, mixed with hydrochloric acid (1 mL, 0.5 N), Arnow's reagent (1 mL) and sodium hydroxide solution (1 mL, 1 N). The absorbance was determined spectrophotometrically at 500 nm. The percentage of phenolic acids, expressed as caffeic acid equivalent on dry weight, was calculated determined using an equation that was obtained from calibration curve of caffeic acid graph (R^2: 0.994083).

2.6 DPPH radical-scavenging activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the tinctures of basil. 1 ml of tincture was added, at an equal volume, to ethanolic solution of DPPH ($0.1gL^{-1}$). After 30 minutes incubation at 40°C in a thermostatic bath, the absorbance was recorded at 517 nm. Synthetic antioxidant, BHT (butylated hydroxytoluene) was used as a standard. The capability of samples to scavenge DPPH· was obtained by comparison of the sample color reduction effect with the control (mixture without working solution) using the following equation and expressed as percentage values: DPPH radical scavenging activity (%) = $[(A_{control} - A_{sample}) / (A_{control})] \times 100$, where $A_{control}$ is the absorbance of DPPH radical+ethanol (containing all reagents except the sample) and A_{sample} is the absorbance of DPPH radical + sample extract/standard [11,12,13].

All absorption spectra were registered using a UV-Vis spectrophotometer Jasco V530.

2.7 Statistical Analysis

All the samples were analyzed in duplicate or triplicate; the average and the relative SD were calculated.

3. Results and discussions

3.1 HPLC-MS results

In this study, 18 phenolic compounds have been investigated by HPLC-MS, in four 50% methanolic extracts of *Ocimum basilicum*. After analysis, 14 phenolic compounds were identified in the extracts (Table 1). They were shown in the order of their retention time; the quantitative determination was performed using the external standard method. The HPLC chromatograms for the four samples of *Basilici herba* are shown in Fig. 1-4.

Two cinnamic acid derivatives, namely caftaric acid (283.566 mg/100 g dried plant) and caffeic acid (82.995 mg/100 g dried plant) were identified and quantified in the extract of sample 1 (*Ocimum basilicum* of Cluj; Table 1, Fig. 1). Gentisic acid, chlorogenic acid, sinapic acid, p-coumaric acid and ferulic acid were also identified, but they were found in low quantities. In the sample 1, the rutin was determined in the largest amount (665.052 mg/100 g dried plant) compared with other samples. The isoquercitrin, and the flavonoid aglycones (luteolin, apigenin, patuletin, quercetol, kaempferol) were identified in low quantities.

In the extract of sample 2 of *Ocimum basilicum* harvested from Dolj, caftaric acid was determined in the largest amount (1595.322 mg/100 g dried plant), followed by caffeic acid (88.452 mg/100 g dried plant). Also, the rutin was identified and quantified in large amount (658.443 mg/100 g dried plant). Gentisic, chlorogenic, p-coumaric, ferulic, sinapic acids, isoquercitrin and the flavonoid aglycons (luteolin, apigenin, patuletin, quercetol, kaempferol) were found in small quantities (Table 1, Fig. 2).

In the extract of commercial sample 3, two free hydroxycinnamic acid derivates, namely caftaric acid and caffeic acid were identified and quantified (Table 1, Fig. 3). Gentisic, chlorogenic, p-coumaric, ferulic, sinapic acids were also identified in the extract of basil, but their concentration was too low to be quantified. In this sample, one flavonoid glycoside, the rutin (quercetin-3-O-rutinoside) was quantified in large amount (388.10 mg/100 g dried plant). Three flavonols (quercetol, kaempferol and patuletin), two flavones (luteolin, apigenin) and isoquercitrin (quercetin 3-glucoside) were detected in a low concentration to be quantified.

In the extract of commercial sample 4, six hydroxycynnamic acid derivates (caftaric, caffeic, chlorogenic, p-coumaric, ferulic, sinapic acids), one dihydroxybenzoic acid (gentisic acid), two quercetol glycosides (rutin, isoquercitrin), three flavonols (quercetol, kaempferol, patuletin) and two flavones (luteolin, apigenin) were detected (Table 1, Fig. 4). The most abundant phenolic acid was caftaric acid (164.185 mg/100 g dry mass) followed by caffeic acid (32.826 mg/100 g dry mass). The gentisic, p-coumaric, ferulic and sinapic acids were found in traces in the extract. In the commercial sample 4, the rutin was found in smaller amount (147.118 mg/100 g dry mass) than in the sample 3 extract (388.105 mg/100 g dried plant).

Considering the 18 standard compounds used in this study, some other peaks were not identified.

For all samples (1, 2, 3 and 4) of *Ocimum basilicum* extracts, the phenolic profile showed the presence of phenolic acid derivatives (caftaric, gentisic, caffeic, ferulic, p-coumaric, chlorogenic, and sinapic acids), the flavonoid glycosides (rutin and isoquercitrin) and free flavonoids (luteolin, apigenin, patuletin, quercetol, kaempferol), in different concentrations. The rutin was determined in high amount in the plants harvested from cultures (samples 1 and 2), two times higher than in the commercial sample 3 and four times more than in the commercial sample 4. Caftaric acid was found in highest quantity in the sample 2.

Table 1: The polyphenolic compounds in extracts of Ocimum basilicum (mg polyphenolic compounds /100 g dried plant)

Polyphenolic compounds	Rt±SD (min)	Sample 1	Sample 2	Sample 3	Sample 4
caftaric acid	2.10±0.06	283.566	1595.322	74.550	164.185
gentisic acid	2.15±0.07	x	x	x	x
caffeic acid	5.60±0.04	82.995	88.452	60.301	32.826
chlorogenic acid	5.62±0.05	x	x	x	x
p-coumaric acid	8.70±0.08	x	x	x	x
ferulic acid	12.20±0.10	x	x	x	x
sinapic acid	14.30±0.10	x	x	x	x
hyperoside	18.60±0.12	-	-	-	-
isoquercitrin	19.60±0.10	x	x	x	x
rutin	20.20±0.15	665.052	658.443	388.105	147.118
miricetol	20.70±0.06	-	-	-	-
fisetin	22.60±0.15	-	-	-	-
quercitrin	23.00±0.13	-	-	-	-
quercetol	26.80±0.15	x	x	x	x
patuletin	28.70±0.12	x	x	x	x
luteolin	29.10±0.19	x	x	x	x
kaempferol	31.60±0.17	x	x	x	x
apigenin	33.10±0.15	x	x	x	x

Notes: -, not found; x, low quantity

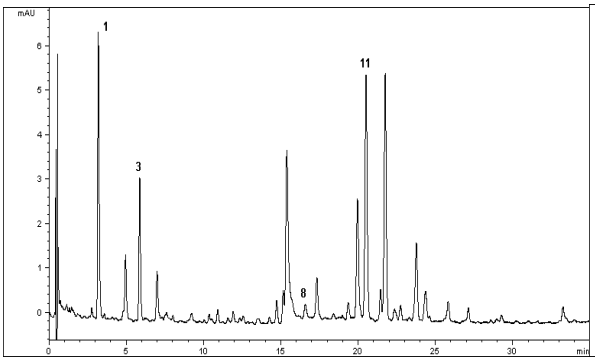

Fig.1: HPLC chromatogram of sample 1

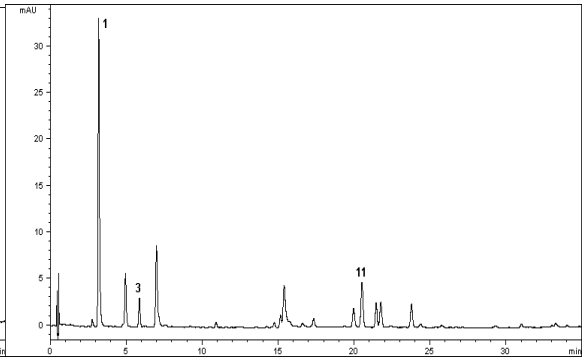

Fig.2: HPLC chromatogram of sample 2

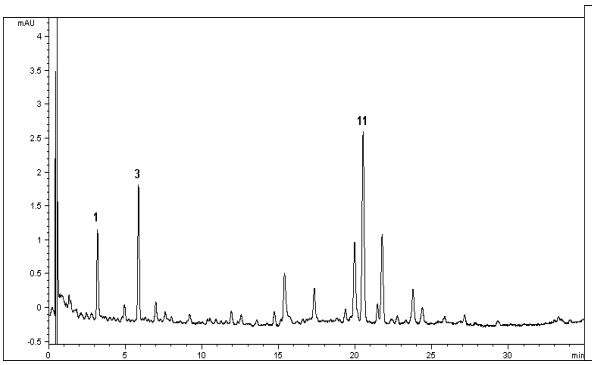

Fig.3: HPLC chromatogram of sample 3

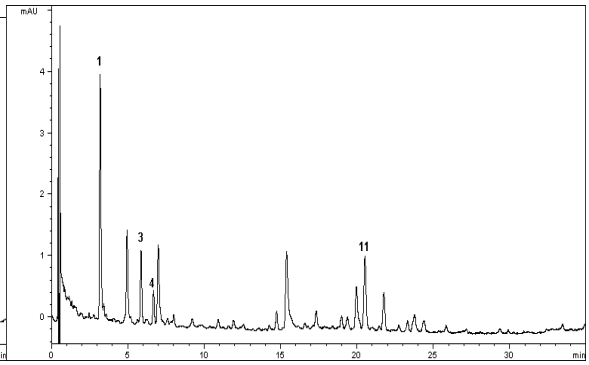

Fig.4: HPLC chromatogram of sample 4

3.2 Determination of polyphenolic compounds content: total polyphenols, flavonoids and caffeic acid derivatives

Natural phenolics are a major group of compounds acting as primary antioxidants or free radical scavengers. Flavonoids and phenolic acids are a ubiquitous group of polyphenolic substances which are present in a lot of plants [4,14,15,16]. Therefore it was also reasonable to determine the total phenolic, total flavonoidic and phenolic acids content in the plant materials (in 50% and 80% ethanolic extracts).

The total phenolic content (TPC) values summarized in Table 2 were quantified based on the linear equation obtained from gallic acid standard calibration curve. Thus, TPC values were expressed as gallic acid equivalent (g GAE/100 g sample). The highest amount of the polyphenols was determined in sample 1, namely in the 80% ethanolic extract (20.335 g GAE/100 g sample), followed by the sample 2 (16.750 g GAE/100 g sample), in the 80% ethanolic extract. The lowest level of polyphenols was detected in samples 3 and 4, namely in the 50% ethanolic extracts (9.120-9.500 g GAE/100 g dry mass). The extraction is a very important stage in the study (isolation, identification, determination) of phenolic compounds. However, there is still no available standardized procedure for simultaneous extraction of all phenolics and applicable to all plant products. For basil, the 80% ethanol is a better solvent for the extraction of polyphenols than 50% ethanol. This may be because many natural polyphenols are soluble in ethanol and less soluble in water [17]. The ethanol had great influence on the extraction: the higher concentration in ethanol (80% aqueous solution of ethanol), resulting in a better extraction of polyphenols (16.750-20.335 g GAE/100 g sample).

The calculation of total flavonoidic content of plant extracts was carried out using the standard curve of rutin and presented as rutin equivalents (mg RE/100 g sample). The flavonoids levels were summarized in Table 3. The obtained values were between 0.350 and 1.245 g RE/dry mass. The samples 1 and 2 were richer in flavonoids (0.970-1.245 g RE/100 dry mass) than samples 3 and 4 (0.450-0.785 g RE/100 dry mass). The amounts of flavonoids were higher in 80% ethanolic extracts (0.450-1.245 g RE/100 dry mass) than in 50% ethanolic extracts (0.350-1.100 g RE/100 dry mass).

The phenolic acids values were expressed as caffeic acid equivalent (g CAE/100 g sample). The results are presented in Table 4. The highest amount of the phenolic acids was determined in 50% ethanol extracts (0.78-2.02 g CAE/dry mass). The sample 1 of *Ocimum basilicum* contains a larger amount of caffeic acid derivatives (2.02 g CAE/dry mass) followed by the samples 2 and 3 (1.58-1.08 g CAE/dry mass). For the commercial sample 4, the level was less than 1% (0.78 g CAE/dry mass).

Table 2: Quantitative determination of total polyphenolic compounds.

Plant samples	TPC (g GAE/100g dry mass)			
	Sample 1	Sample 2	Sample 3	Sample 4
O. basilicum - 50% ethanol extract	14.975± 0.992052	12.720± 1.667573	9.500± 1.136515	9.120± 1.017153
O. basilicum - 80% ethanol extract	20.335± 0.998282	16.750± 1.260952	11.200± 0.962635	10.990± 0.795864

Table 3: Quantitative determination of flavonoidic compounds.

Plant samples	Flavonoids (g RE/100 dry mass)			
	Sample 1	Sample 2	Sample 3	Sample 4
O. basilicum - 50% ethanol extract	1.100± 0.266958	0.862± 0.210466	0.575± 0.08226	0.350± 0.088318
O. basilicum - 80% ethanol extract	1.245± 0.459456	0.970± 0.072572	0.785± 0.076811	0.450± 0.138323

Table 4: Quantitative determination of caffeic acid derivatives

Plant samples	Caffeic acid derivatives (g CAE/dry mass)			
	Sample 1	Sample 2	Sample 3	Sample 4
O. basilicum - 50% ethanol extract	2.02± 0.180185	1.58± 0.297097	1.31± 0.105515	0.78± 0.133666
O. basilicum - 80% ethanol extract	1.84± 0.124097	1.08± 0.187794	1.05± 0.177951	0.61± 0.053541

The sample 1 of *Ocimum basilicum* harvested of Cluj contains a highest amount of polyphenolic, flavonoidic compounds and phenolic acids, followed by the sample 2 of basil harvested from Dolj. The two commercial samples (3 and 4) contain up to half of the values obtained for harvested samples (samples 1 and 2). The presence of active principles depends on a number of factors including the plant species, genetic factors, geographical location, differences in growth, the type of soil, the time and season of harvest, the way the herb is prepared, drying, and storage etc. A well-defined and constant composition of the product is therefore, one of the most important prerequisites for the production of a quality drug [18].

3.3 In-vitro antioxidant activity

Determination of the free radical scavenging activity of plant extracts

The antioxidant capacity was tested by the DPPH method, BHT was used as standard control and the capacity of the samples to scavenge DPPH· was expressed as percentage values (Table 5). The radical scavenging activity in the plant extracts decreased in the following order: sample 1 > sample 2 > sample 3 > sample 4. The radical scavenging effect of the harvested samples of *Ocimum basilicum* at a concentration of 0.4 mg plant product/mL extract was between 22.43 and 26.55%, followed by the commercial samples (11.24-12.05%) at the same concentration. The highest radical scavenging activity was showed by BHT, a synthetic

antioxidant (94.77%±0.646735) at the same concentration (0.4 mg mL^{-1}). The results showed that the basil harvested of cultures possessing a higher level of polyphenolic compounds had superior antioxidant capacities compared to the commercial samples. The antioxidant effect of the samples 1 and 2 was two times greater than that of the commercial samples. Although we detected a large amount of phenolic content in *O. basilicum*, it did not show a significant antioxidant potential by DPPH scavenging method. These results show that even if the phenolic compounds play an important role as antioxidants, in this survey their activity was moderate. The high concentration of polyphenols may be correlated to the anti-inflammatory, antimicrobial or diuretic properties due to which *Ocimum basilicum* is used in therapy [1,19]. Our results were similar to those obtained for Malaysian *Ocimum basilicum*, determined by the same method [20]. Generally, in previous studies, the research topic led to the same conclusion: the antioxidant activity of basil is moderate, even if the polyphenolic content is appreciable [5,20].

Table 5: Results of DPPH free radical scavenging

Samples	DPPH Radical Scavenging Activity (%)
BHT (standard control)	94.77±0.646735
Sample 1	26.55±0.775672
Sample 2	22.43±0.6063
Sample 3	12.05±0.843603
Sample 4	11.24± 0.898387

4. Conclusions

The results of the study suggest that all extracts of *Ocimum basilicum*, especially the extracts of samples harvested from cultures (samples 1 and 2) contain a large amount of total polyphenolic compounds, including flavonoids and caffeic acid derivatives. The antioxidant effect of the harvested samples was higher than the activity of the commercial samples. Nevertheless, all the samples of *O. basilicum* showed moderate scavenging effects against DPPH radical, proportionate to the content of phenolic compounds. The presence of polyphenols in *Ocimum basilicum* can be related to diuretic or anti-inflammatory properties.

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