ORIGINAL PAPER

QUANTITATIVE ANALYSIS OF POLYPHENOLS AND ANTIOXIDANT ACTIVITY OF MINT MACERATE

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Abstract. *The total phenols content of alcoholic extractive solution of mint (Mentha piperita) was examined using Folin-Ciocâlteau method and the composition of its phenolic components was investigated by adapted USP30 HPLC method. The analyzed mint macerate exhibits high total phenolic content amoung which three major phenolic compounds were identified: gallic acid, chlorogenic acid and caffeic acid. The antioxidant activity of Mentha alcoholic extractive solution was evaluated using DPPH Radical Scavenging test.*

Keywords: *mint, antioxidant activity, total phenolic compounds, DPPH.*

1. INTRODUCTION

Mentha is the most important genus in the *Labiatae* family because it is a precious source of essential oils with high economic value [1]. All species of Mentha are essential oil bearing and they are distributed across Europe, Asia, Africa, North America and Australia [2, 3]. Mint oils are produced in different parts of the world and the most economical valuble are: cornmint (the source of natural menthol), peppermint, Scotch spearmint and Native spearmint [1, 4, 5]. The amount of Mint oils produced annually is about 23,000 metric tonnes with an economical value estimated at $400 million [1, 5, 6, 7]. Menthol is used as a raw material in perfumery, confectionary, tobacco industry and pharmaceutical preparations. About 40% of the total mint oil consumption is found in tobacco industry followed by pharmaceutical and confectionary industries [3, 7].

Besides its industrial and culinary uses, mint is also used in traditional systems of medicine. Peppermint oil is one of the world's oldest herbal medicines. Mints are mainly used to cure gastrointestinal disorders, but the spectrum of medical activities is larger [1, 3, 8, 9]. It is also recognized for its carminative, stimulant, antispasmodic, antiseptic, anti-infammatory, antibacterial and antifungal activities [3, 10-14]. Studies showed that generally, Gram-negative bacteria are more resistant to mint essential oil than Gram-positive bacteria, which in some particular cases represents an alternative [11, 12] to other synthetic compounds with antibacterial activity [11-16].

Although some healthcares believe that herbal medicines, such as the essential oil from *Mentha* sp., are safe, relatively recent publications have reported potentially severe side effects [3, 17]. Spearmint and peppermint tea can cause iron deficiency anemia [3].

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Potentially toxic compounds in peppermint are pulegone and menthol which can cause heartburn, nausea, vomiting, allergic reactions, flushing, and headaches [3]. According to the Committee of Experts on Flavoring Substances (CEFS), provisional consumption limits were established for pulegone at 20 mg/kg in food and beverages [18]. Peppermint oil is contraindicated in obstruction of the bile ducts, gallbladder inflammation, and severe liver failure [3, 18].

Plants which are either naturally grown or cultivated in heavy metal contaminated regions have high metal accumulations which cause serious health damage to human and livestock [19-23]. Heavy metals causes oxidative stress in plant which results in a variety of harmful effects in plant cells, such as inhibition of photosynthetic activity, lipid peroxidation (MDA) and DNA damage. Therefore it is important to have a heavy metal monitorided culture or to provide an organic culture for food and medicinal plants [24-32]. In peppermint essential oil were identified more than 300 compounds. The terpenic class is the most represented (61%), whereas other groups, such as aldehydes (9%), aromatic hydrocarbons (9%), miscellaneous (8%), lactones (7%), and alcohols (6%), have been shown to be present in a smaller proportion [1, 3, 33-37].

Species of the genus *Mentha* have been reported to contain a range of components such as: phenolic acids (e.g., rosmarinic and caffeic acids), flavones (e.g., luteolin derivatives), and flavanones (e.g., eriocitrin derivatives). They are possibly the major antioxidants [28-30]. Regarding phenolic acids, the genus *Mentha* is particularly rich in caffeic acid and its derivatives, chlorogenic and rosmarinic acid [1, 3, 36-40], 60–80% of total phenolic compounds [3]. Among these phenolic acids, seven salvianolic acids have been described in *Mentha* plants [3, 26]. Vitamins reported in mint (e.g., ascorbic acid and carotenoids) are minor contributors to the overall antioxidant potential, but is an excellent source of, which is converted to vitamin A in the body [3, 38-44].

Based on these considerations, the objectives of this study were to characterise the alcoholic macerate of local *M. piperita* by evaluating the pH, ORP, the antioxidant capacity and total phenolic content using the Folin-Ciocâlteau spectrophotometric method and also to investigate the composition of its phenolic components using HPLC-DAD method.

2. MATERIALS AND METHODS

2.1. MATERIALS

Gallic acid was purchased from Fluka (Buchs, Switzerland) and Folin-Ciocâlteau reagent from Merck (Darmstadt, Germany). Gallic acid (standard phenolic compound) 1×10^{-2} mol$\times$L^{-1} was prepared by dissolving 0.1881g of gallic acid in 100 mL of ethanol. Folin-Ciocâlteau reagent was diluted with distilled water 1:2 (V:V).

DPPH (2,2-difenil-1-picrililhidrazil) was purchased from Aldrich (Germany). The standard compound solution 0.0063% (1.268 mM) was prepared in a 200 mL calibrated flasks by dissolving 0.001g of 2,2-difenil-1-picrililhidrazil in methanol. All used reagents were of HPLC reagent grade. Spectrometric measurements were carried out using a UV-VIS JASCO 550 scanning spectrophotometer.

2.2. *METHODS*

Extraction of polyphenols

Aerial part of mint (*Mentha piperita*) was collected from organic culture during July 2018 in Topraisar, Constanta, Romania. Extractions were achieved by maceration of 50 g of fresh plant with 200 mL ethanol (p.a, Merck) at room temperature for 7 days. The mixtures were kept at dark and strongly shaken two times every day. The sample was separated by filtration and immediately analysed. The pH and oxidation potential (ORP) were measured with pH-300 Portable Meter.

Total polyphenolic content (TPC)

The total phenols were estimated according to the Folin-Ciocâlteau method as previously described [18, 25-27, 29, 34, 35]. Total phenolic content was determined using the spectrophotometric method based on the reduction of a phosphowolframate – phosphomolibdate complex to blue products by soluble phenolic compounds, in sodium carbonate media and the measurement of the absorption of the formed complex at the wavelength of 681 nm. The absorbance relative to a gallic acid standard curve was measured and results are expressed as gallic acid equivalents (mg GAE/mL). All samples were performed in triplicate and the mean value was reported. The spectrometric measurements have been performed using a JASCO V550 spectrophotometer.

Calibration curve

For calibration curve volumes of 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL and 7 mL of gallic acid standard solution were used. To plot the calibration curve in the range of 0.68 – 4.76 mg GAE/L, 1 mL Folin-Ciocâlteau reagent 1:2 was added in 50 mL calibrated flasks to different volumes of standard gallic acid solution, then 1 mL sodium carbonate solution 20%; after 10 minutes the volume was made up to 50 mL with distilled water. Each sample was homogenized and let 30 minutes at room temperature for the colour stabilization and after that the absorbance was read at 681 nm (Fig. 1). The correlation coefficient was 0.9976.

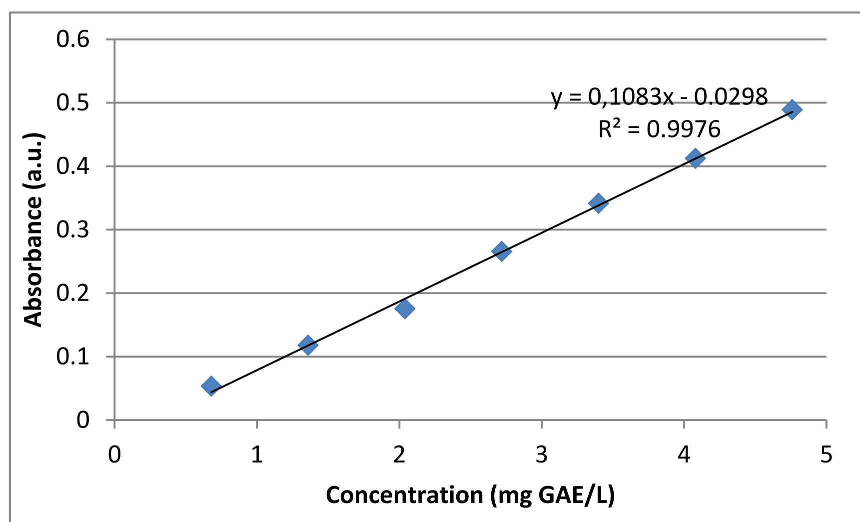

Figure 1. Calibration curve of gallic acid in the range of 0.68 – 4.76 mg GAE/L at 681 nm.

The characteristics of the obtained calibration curve are: Y = A + B* X; A = 0.0298; B = 0.1083 and Correlation Coefficient = 0.9976. To measure the total phenols content, 2 mL of diluted sample (1/250) was added in 50 mL calibrated flasks, then 1 mL Folin-Ciocâlteau reagent 1:10, 1 mL sodium carbonate solution 20% and the process was the same like those used for calibration.

HPLC analysis of phenolic compounds

The resulted extractive solution was analysed by an adapted USP30 HPLC method used for separation, identification and quantification of the phenolic compounds as previously described [18, 30-32, 38-40, 45].

For separation there was used a Zorbax Eclipse XDB-C18 column: 250 mm, 4.6 mm; 5 µm (Agilent Technologies). The gradient elution was phosphoric acid 0.1% in water (solvent A) and acetonitrile (solvent B) as presented in Table 1.

Table 1. The gradient of elution solvents

Time (min.)	Solvent A, %	Solvent B, %
0-13	90	10
13	78	22
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

The operation parameters of chromatographic process were: the flow rate - 1.5 mL/min; the injection volume - 20 µL; the analysis time - 22 minutes. Quantification of sample compounds was at 310 nm and 35°C. The retention times and DAD spectra were compared to available authentic standards. To register the retention time of standard a mixture of standard solutions in 70% methanol having the following the concentrations it was used: E – resveratrol = 37 mg/mL, Z – resveratrol = 0.22 mg/L, caffeic acid = 0.36 mg/mL, chlorogenic acid = 0.37 mg/mL, cinnamic acid = 0.58 mg/mL, vanillin = 0.42 mg/mL, gallic acid = 0.39 mg/mL, ferulic acid = 0.48 mg/mL, 3-methylgalic acid = 0.34 mg/mL, ellagic acid = 0.43 mg/mL, *p*-coumaric acid = 0.51 mg/mL (Table 2).

Standard deviations of retentions time were obtained after statistical processing of the 6 injections (soft SPSS 10). The retention times were between 0.990±0.025 minutes for gallic acid and 15.867±0.007 minutes for cinnamic acid. Identification and quantitative determination of the active constituents from sample extract was done by comparing the chromatogram of standards mixture.

Table 2. The retention time of standards

Standards	**Retention time** ± SD
gallic acid	0.990 ± 0.025
3-*o*-methylgallic acid	2.606± 0.008
chlorogenic acid	3.501 ± 0.015
caffeic acid	4.598 ± 0.036
vanillin	6.919 ± 0.051
p-coumaric acid	7.187± 0.019
ferulic acid	8.565± 0.058
E - resveratrol	14.467 ± 0.017
ellagic acid	15.303± 0.027
Z - resveratrol	15.751 ± 0.058
cinnamic acid	15.867 ± 0.007

**standard deviation for six injections*

DPPH Radical Scavenging test

The antioxidant capacity was evaluated using DPPH Radical Scavenging test. Gallic acid (GA) was used as standard to plot calibration curves and the results were expressed as equivalents (mg GAE) [18, 27, 33, 45-47]. In 25 mL calibrated flasks different volumes of gallic acid solutions were added, then 5 mL DPPH 0.063% (1.268 mM) in methanol, filled up to the mark with methanol and let in the dark, at room temperature for 45 minutes before the absorbance registration at 530 nm versus methanol. Previously, the DPPH solution spectrum was recorded and the maximum absorbance was registered at 530 nm using a JASCO V550 spectrophotometer. The solutions absorbances decrease due to the antioxidant capacity of standard compounds determined the downward allure of calibration curves.

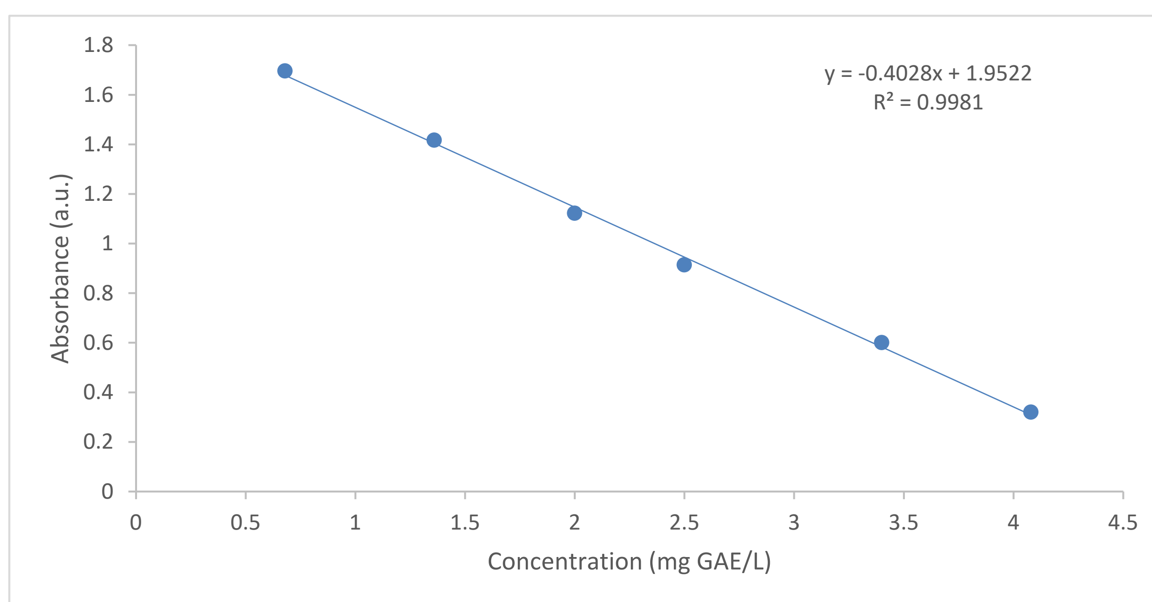

Figure 2. Calibration curve of gallic acid in the range of 0.50 – 4.00 mg GAE/L at 530 nm.

The calibration curve with gallic acid as standard was linear in 0.50 – 4.00 mg GAE/L range and the correlation coefficient was 0.9981. To measure the antioxidant capacity, 1 mL of diluted sample (1/250) was added in 25 mL calibrated flasks, then treated with 5 mL DPPH 1.268 mM in methanol, filled up to the mark with methanol and let in the dark, to the room temperature for 45 minutes before the absorbance registration at 530 nm using methanol as blank.

3. RESULTS AND DISCUSSION

3.1. RESULTS

In Table 3 are presented the values of pH, ORP, total phenolic compounds determined by Folin-Ciocâlteau method and value of DPPH Radical Scavenging test of investigated macerate of Mint (*Mentha piperita*).

Table 3. Physico chemical parameters, total phenols and antioxidant activity values of mint (*Mentha piperita*) macerate.

Parameter, UM	**Value**
pH, units	6.21
ORP, mV	61
Total phenols, mg GAE/100g fresh weight	1560
DPPH, mg GAE/100g fresh weight	5472

Table 4. Contents [mg/100g f.w.] and percentages [%] of phenolic compounds in mint macerate determined by HPLC-DAD.

Phenolic compound	***Mentha piperita* fresh product macerate**	
	mg/100g f.w.	**%**
Gallic acid	18.6	71.93
3-*o*-Methyl-gallic acid	-	-
Feluric acid	-	-
Ellagic acid	-	-
p-Coumaric acid	-	-
Trans-Resveratrol	-	-
Chlorogenic acid	2.46	9.51
Caffeic acid	4.8	18.56
Caftaric acid	-	-
Cinnamic acid	-	-
Total Phenols	**25.86**	**100**

In Table 4 there are shown the values of phenolic compounds founded in the investigated mint extract by HPLC and compared to available authentic standards.
The HPLC values were used to calculate the percentages (%) of each founded phenolic compound as well as the sum of total phenols.

In Fig. 3 (a-b) are presented the HPLC chromatograms of the investigated mint extract.

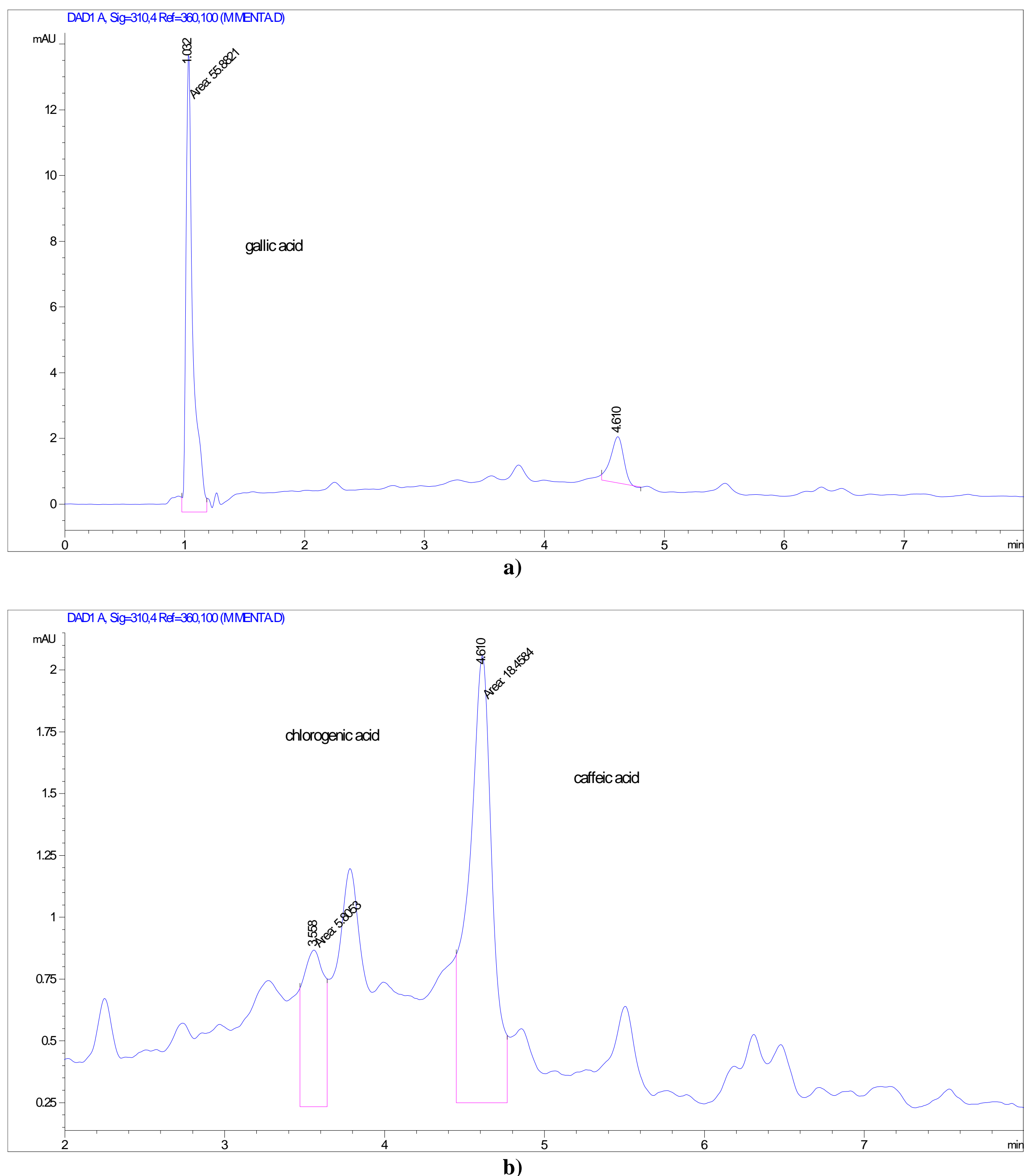

Figure 3. HPLC chromatogram of Mint (*Mentha piperita*) extract

3.2. DISCUSSION

The value of total phenols content in mint determined by Folin-Ciocâlteau method is high (1560 mg GAE/100 g fresh weight) and comparable to other literature data [1, 3, 33].

The analysed *Mentha piperita* macerate exhibits high DPPH Radical Scavenging test value (5472 mg GAE/100g fresh weight) which indicates that antioxidant capacity is given not only by the phenolic content but also by other major components existing in the studied macerate.

Compare to the available authentic standards used for determinations, only three individual polyphenolic compounds were found; gallic acid was in the highest concentration. Caffeic acid was the second major compound determined in the analysed sample with values comparable to the reported literature data [3]. Small amount of chlorogenic acid is also present in the mint macerate.

All data confirm that the antioxidant activity is due not only to the presence of phenolic compounds found in sample but also to other compounds with major contribution to the overall antioxidant potential.

4. CONCLUSIONS

Mentha piperita macerate collected from organic culture was analyzed using electrometric methods (pH and ORP), molecular spectrometric methods (total phenols content by Folin-Ciocâlteau, DPPH Radical Scavenging test to establish the antioxidant capacity value). The individual phenolic components were investigated using HPLC-DAD method and compared to available authentic standards.

The obtained data indicate that the content of total phenolic compounds is high and comparable to the literature data. Gallic acid and caffeic acid are the major compounds determined in the analyzed sample using HPLC-DAD method. The antioxidant activity value is higher than the total phenols value which is due to the presence of other compounds with major contribution to the overall antioxidant potential. All data indicates that *Mentha piperita* is a proper antioxidant source which can be used in diets or to get other health benefits.

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