Ultrasound-assisted extraction of bioactive compounds from lemon balm and peppermint leaves

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A b s t r a c t. The aim of this study was to investigate the influence of conventional and ultrasound-assisted extraction (frequency, time, temperature) on the content of bioactive compounds as well as on the antioxidant activity of aqueous extracts from fresh lemon balm and peppermint leaves. Total phenols, flavonoids, non-flavonoids, total chlorophylls, total carotenoids, and radical scavenging capacity were determined. Moreover, the relationship between bioactive compounds and antioxidant capacity was studied by linear regression. A significant increase in all studied bioactive compounds during ultrasonic extraction for 5 to 20 min was found. With the classical extraction method, the highest amounts of total phenols, flavonoids, and antioxidant activity were determined, and the maximum amounts of total chlorophylls and carotenoids were determined during 20 min ultrasonic extraction. The correlation analysis revealed a strong, positive relationship between antioxidant activity and total phenolic compounds.

K e y w o r d s: total phenol content, chlorophylls, antioxidant activity, conventional extraction, high intensity ultrasound

INTRODUCTION

Phenolic compounds exhibit a high degree of degradation in terms of technological processes and show distinct thermolability, sensitivity to light, the impact of pathogens, mechanical damage to plant tissue cells (Ross *et al.*, 2009). Lately modern, non-invasive extraction techniques of plant metabolites with the main objective of increased extracted compound yield and a shortened period of extraction have been increasingly popularized. One of the mentioned techniques is high-intensity ultrasound, which shows a wide range of application in the technological processes *eg* inactivation of microorganisms, crystallization, filtration, drying, extraction, homogenization, stimulation of oxida-tion, emulsifying (Barba *et al.*, 2015a; Deng *et al.*, 2015; Herceg *et al.*, 2009; Koubaa *et al.*, 2015). High intensity ultrasound treatment does not show any degradation rates on the content of bioactive compounds in the treated food products (Koubaa *et al.*, 2015; Šic Žabur *et al.*, 2015; Zinoviadou *et al.*, 2015). In addition, high intensity ultrasound is applicable for extraction of compounds with various chemical structures and is characterized by non-invasive temperatures (Cvjetko Bubalo *et al.*, 2013; Koubaa *et al.*, 2015; Šic Žlabur *et al.*, 2015). The ultrasound technique is extremely simple in application, and usually two types of equipment are used, including:
– a system with a directly immersed ultrasonic probe (transducer)
– an ultrasonic bath.

Phenolic acids, flavonoids, and tannins are the most commonly found polyphenolic compounds in plant extracts (Naik *et al.*, 2006). Phenolic compounds from plant material have recently gained great popularity because of their significant positive effects on human health (Auclair *et al.*, 2008; Barba *et al.*, 2014). Phenolic compounds along with plant pigments (carotenoids, chlorophylls) have proven significant antioxidant activity, which is directly corelat-ed with removal of free radicals and superoxide (Hsu *et al.*, 2013; Sinkovič *et al.*, 2015). Therefore, it is import-ant to emphasize the need to increase the consumption of foodstuffs rich in phenolic compounds aimed at preventive action on human health from cancer disease prevention to occurrence and prevention of cardiovascular diseases (Barba *et al.*, 2014).

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Peppermint (*Mentha piperita* L.) and lemon balm (*Melissa officinalis* L.) are mainly used as a spice in many food products (De Vincenzi *et al.*, 1991) and traditionally used for medical purposes (Foster and Tyler, 1993). Peppermint and lemon balm are plant species characterized by a high content of phenolic compounds, mainly flavonoids (Guédon and Pasquier, 1994) but they are also rich in chlorophylls. A number of positive effects of peppermint on human health are known, such as antibacterial (Hajlaoui *et al.*, 2008), antifungal (Bouchra *et al.*, 2003), and insecticidal activity (Saljoqi *et al.*, 2006). Lemon balm is one of the most popular medicinal plants with proven sedative, antispasmodic, antibacterial, antiviral, anti-inflammatory, and antioxidative effect (Yamasaki *et al.*, 1998).

MATERIALS AND METHODS

Peppermint and lemon balm were cultivated on the experimental field of the Department of Vegetable Crops, Faculty of Agriculture University of Zagreb. The experiments were performed in triplicate. During plant cultivation, no fertilization treatment was applied. Also, the availability of water to plants was exclusively dependent on climatic conditions; irrigation was not applied on the experimental field. Peppermint and lemon balm plants were harvested with the whole stem in mid-June 2014. Before analysis, only green, healthy leaves with no visible signs of damage were torn off from the plant stems, while the stem and leaf petiole were removed. The leaves were washed under cold running water and dried. All chemical analyses were made on leaves and leaf water extracts in triplicate.

The water extracts of plant leaves for each plant material, peppermint and lemon balm, were prepared with a unique procedure: 2 g (±0.01) of plant leaves were cut into smaller pieces and weighed in an Erlenmeyer flask with a volume of 100 ml, onto which 50 ml of distilled water at room temperature (22°C) were added. Prepared leaf solutions were allowed to stand for 24 h at room temperature

with intermitted shaking. After 24 h, the solutions were filtered through Whatman filter paper and used for the designed chemical analysis.

An ultrasonic bath (Bandelin, RK 103 H, Germany) with frequency 35 kHz and nominal power 140 W was used for ultrasound extraction. Peppermint and lemon balm water solutions were prepared with a unique procedure: 2 g (±0.01) of fresh plant leaves were weighed in an Erlenmeyer flask with a volume of 100 ml, onto which 50 ml of distilled water at room temperature (22°C) were added. During the ultrasonic treatment, the time of extraction was 5, 10, 15, 20, 25, and 30 min. After each ultrasonic extraction, solutions were filtered through Whatman filter paper and were used for the designed chemical analysis. During the sonication of the samples, temperature change was measured after 5, 10, 15, 20, 25, and 30 min with an infrared thermometer (Batronix, IC UT-300B, Germany) (Table 1).

The total phenol contents in peppermint and lemon balm leaves (raw material) were determined with the Ough and Amerine (1998) method. In turn, the total phenolic content of peppermint and lemon balm extracts (aqueous solutions) were obtained according to Shukla *et al.* (2012) with some modification during the analysis. Measurement was carried out as follows: 1 ml of the prepared aqueous extract and 1 ml of the Folin-Ciocalteu reagent diluted with distilled water (1:2) were added in the volumetric flask with a volume of 50 ml and allowed to stand for 3 min. Additionally, 3 ml of a saturated sodium carbonate solution was added, the flask was filled to the mark with distilled water and allowed to stand for 3 h at room temperature with intermittent shaking. The absorbance of the blue colour that developed was measured spectrophotometrically (Shimadzu UV 1650 PC) at 750 nm with distilled water as a blank. The flavonoid content of the peppermint and lemon balm extracts were determined with the Abou-Arab and Abou-Salem (2010) method with some modification during the analysis: 1 ml of

T a b l e 1. Experimental conditions

Extraction technique	Solvent	Solvent volume (ml)	Time	Temperature (°C)	Ultrasonic bath
Conventional	Distilled water	50	24 h	22.1	–
UAE	Distilled water	50	5 min	28.2	35 kHz/140 W
			10 min	40.3	
			15 min	46.0	
			20 min	54.2	
			25 min	54.7	
			30 min	56.4	

UAE – ultrasound assisted extraction.

the prepared aqueous extracts, 1 ml of 20% (v/v) HCl, and 0.5 ml formaldehyde were added in the volumetric flask with a volume of 25 ml. The prepared samples were purged with nitrogen (N_2) and allowed to stand for 24 h at room temperature. After 24 h, the same Folin-Cioacalteu reaction as for total phenols was performed. The absorbance of the blue colour that developed was measured spectrophotometrically (Shimadzu UV 1650 PC) at 750 nm with distilled water as a blank. The non-flavonoid content was expressed mathematically as the difference between total phenols and flavonoids. Gallic acid was used as an external standard and the concentration of total phenols, flavonoids, and non-flavonoids was expressed as mg g^{-1} of the aqueous extract.

The content of total chlorophylls (chlorophyll a and chlorophyll b) and total carotenoids were determined by Holm (1954) and Wettstein (1957). The sample was weighed into a test tube and 10 ml of acetone was added.The solution prepared in this way was homogenized with a laboratory homogenizer (IKA, UltraTurax T-18, Germany). The solution was filtered and transferred into a volumetric flask with a known volume. The absorbance was measured spectrophotometrically (Shimadzu UV 1650 PC) at 662, 644, and 440 nm with acetone as a blank probe. The absorbance values were recalculated according to the Holm-Wettstein equations in the pigment concentration (mg l^{-1}), and the final result of the pigment content was expressed in mg g^{-1}:

$$\text{chlorophyll a} = 9.784\,A_{662} - 0.990\,A_{644},$$

$$\text{chlorophyll b} = 21.426\,A_{644} - 4.65\,A_{662},$$

$$\text{chlorophyll a+b} = 5.134\,A_{662} + 20.436\,A_{644},$$

$$\text{carotenoids} = 4.695\,A_{440} - 0.268\,(\text{chlorophyll a+b}).$$

The antioxidant activity of leaves and aqueous extracts of peppermint and lemon balm leaves was determined with the ABTS method. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; SigmaAldrich) was used as an antioxidant standard. Trolox (2.5 mM) was prepared in ethanol (80 %) for use as a stock standard. ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), and potassium persulfate were obtained from Sigma-Aldrich. The experiments were performed according to Miller *et al.* (1993). ABTS (7 mM) and potassium persulfate (140 mM) were dissolved in distilled water. These two solutions were mixed (88 µl of prepared potassium persulfate (140 mM) and 5 ml of prepared ABTS solution) and the mixture was allowed to stand in the dark at room temperature for 16 h before use in order to produce ABTS radicals (ABTS•+). On the day of the analysis, 1% ABTS•+ solution (1 ml ABTS•+ in a volumetric flask of 100 ml filled with 96% ethanol to the mark) was prepared. For the study, the absorbance of the ABTS radical solution was measured at 734 nm and set to absorbance of 0.70±0.02. 160 µl of the extracts obtained were directly injected in the cuvette, mixed with

2 ml 1% ABTS•+, and absorbance was measured at 734 nm. The final results of the antioxidant activity was calculated based on the calibration curve and expressed as mmol TE l^{-1}.

Statistical analysis was performed using the SAS® version 9.3. Data were subjected to the one-way analysis of variance (ANOVA) according to the treatment structure. Mean values were compared by the t-test (LSD), and they were considered significantly different at p≤0.05. Regression and correlation analysis were performed in order to investigate the nature and intensity of the relationships between bioactive compounds and antioxidant activity in vitro. Correlation coefficients with p values ≤0.05 were considered significant.

RESULTS AND DISCUSSION

A high statistically significant difference (p≤0.0001) was observed for all the analysed chemical compounds depending on the extraction technique (Table 2). The amount of total phenolic compounds in fresh lemon balm leaves was 1 977.45 mg l^{-1}. Many literature data indicate a high content of phenolic compounds in lemon balm leaves (Dastmalchi *et al.*, 2008; Komes *et al.*, 2011; Najafian and Rowshan, 2013). The aqueous samples of lemon balm leaves extracted with the classical method for 24 h at room temperature show a high content of phenolic compounds with the determined total phenol content of 1331.54 mg l^{-1}. The aqueous samples of lemon balm leaves extracted by high intensity ultrasound in an ultrasonic bath show a significant increase in phenolic compounds depending on the extraction time. The content of all the phenolic compounds studied show an increasing trend in the time of ultrasonic extraction from 5 to 25 min. During the period mentioned, the total phenol content increased by about 6.5 times. The highest value of the total phenolic content in the samples treated with ultrasound was determined in UES5 during 25 min of extraction (Table 2), while sample UES6 (extraction time 30 min) had even a lower content of phenolic compounds than sample UES4. The increasing trend of the total phenol content during the ultrasonic treatment for 30 min is expected, since the extraction time plays a key role in the separation of chemical compounds (Koubaa *et al.*, 2015; Šic Žlabur *et al.*, 2015). The results of total phenols extracted with the classical method show higher yields compared to the phenol results obtained in samples treated with ultrasound. In aqueous samples of lemon balm leaves extracted classically, only 1.5-fold higher total phenol content was determined compared to sample UES5. During the classical extraction, even 24 h was required to extract the high content of phenolic compounds. High-intensity ultrasound shows significantly better results compared to conventional extraction because already during 25 min of application thereof a very high content of phenolic compounds (891.76 mg l^{-1} of extract) was achieved. The results obtained are in agreement with other literature data

T a b l e 2. Total phenol, non-flavonoid, flavonoid content and antioxidant capacity of leaves and aqueous samples of lemon balm leaves extracted with utrasound and classical methods

Extraction technique	Total phenols	Non-flavonoids	Flavonoids	Antioxidant capacity
	(mg l^{-1} extract)			(mmol TE l^{-1})
Leaves	1977.45±4.53 a	1739.46±1.335 a	237.99±0.45 d	2.42±12.77 a
Classical extraction	1331.54±0.575 b	750.90±1.82 b	580.64±1.25 a	2.29±16.5 b
UES1	138.11±0.765 h	80.31±1.45 h	57.79±1.36 h	1.91±5.8 e
UES2	225.92±0.45 g	144.82±0.45 g	81.10±0.02 g	1.99±3.0 ed
UES3	310.86±0.07 f	210.40±0.065 f	100.46±0.45 f	2.09±15.3 cd
UES4	720.93±1.92 d	500.16±1.92 d	213.02±0.6 e	2.10±16.86 cd
UES5	891.76±0.825 c	507.92±0.065 c	391.61±1.255 b	2.17±4.15 c
UES6	637.97±0.45 e	290.36±0.125 e	347.61±0.355 c	2.05±3.2 d

UES1, UES2, UES3, UES4, UES5, UES6 – ultrasound extracted samples: 5, 10, 15, 20, 25, and 30 min, respectively. Different letters indicate significant differences between means at p<0.0001.

(İnce *et al.*, 2013). The antioxidant activity is in correlation with the total phenolic content, vitamins, and generally bioactive compounds (Barba *et al.*, 2010, 2013; Carbonell-Capella *et al.*, 2014; Criado *et al.*, 2014). According to the results obtained in this study, a strong relationship between antioxidant activity and the content of total phenolic compounds, flavonoids, and non-flavonoids in the aqueous samples of fresh lemon balm leaves extracted with the classical and ultrasonic methods was determined (Table 3). The highest antioxidant activity was determined in a sample of fresh lemon balm leaves (2.42 mmol TE l^{-1}) that also contains the highest amount of all the studied phenolic compounds. The antioxidant activity was the lowest in the aqueous extract of lemon balm leaves treated with ultrasound for 5 min (UES1), in which the total phenol content was the lowest. The increasing trend of antioxidant activity was determined for samples UES2, UES3, UES4, and UES5, while, as expected, sample UES6 showed the lowest antioxidant activity. The aqueous samples of lemon balm leaves extracted classically showed high antioxidant activity. Generally, lemon balm is exceptionally rich in phenolic compounds. The results of this research indicate a high content of phenolic compounds in all the studied aqueous extracts of lemon balm leaves as well as strong antioxidant activity and are in line with other literature data (Caniova and Brandsteterova, 2001; İnce *et al.*, 2013; Karasová and Lehotay, 2006).

The linear regression between the antioxidant activity and the total phenol content of lemon balm aqueous extracts is presented in Fig. 1a. The total phenol content of the lemon balm aqueous extracts was found to correlate significantly with the antioxidant activity (ABTS). In this research, the relationship between the antioxidant activity

T a b l e 3. Correlation coefficients (r) between the selected chemical compounds and antioxidant activity (mmol TE l^{-1}) in different aqueous extracts of lemon balm and peppermint leaf samples

Lemon balm aqueous extracts	Antioxidant activity
Total phenols	0.932***
Total flavonoids	0.869 **
Non flavonoids	0.938***
Total chlorophylls	0.444 NS
Chlorophyll a	0.380 NS
Chlorophyll b	0.471 NS
Total carotenoids	0.800*
Peppermint aqueous extracts	
Total phenols	0.874**
Total flavonoids	0.961***
Non flavonoids	0.836**
Total chlorophylls	0.019 NS
Chlorophyll a	-0.078 NS
Chlorophyll b	0.091 NS
Total carotenoids	0.549 NS

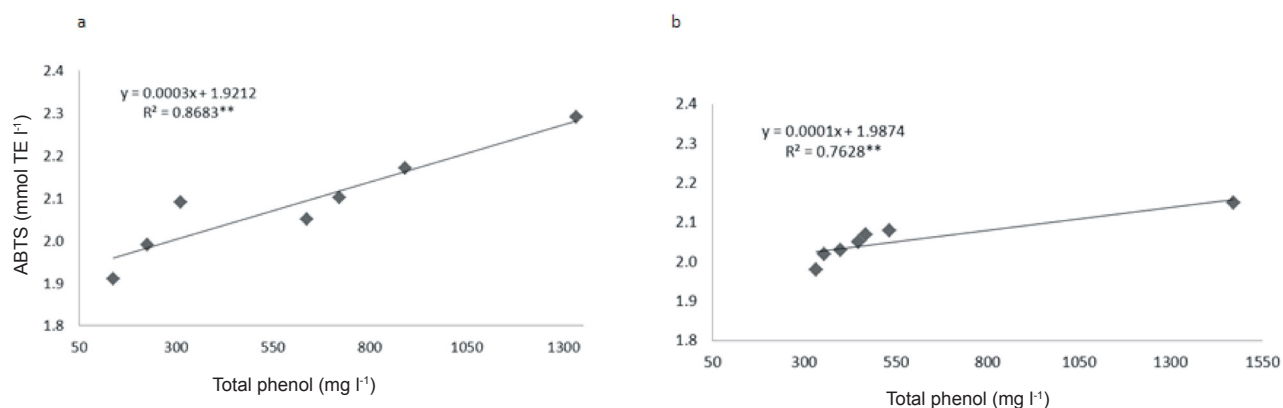*0.01<p<0.05, **p<0.001, ***p<0.0001, NS – not significant.

Fig. 1. Linear regression between total phenol content (mg l⁻¹) and antioxidant activity (mmol TE l⁻¹) in aqueous extracts of: a – lemon balm and b – peppermint leaf samples. The significance of the correlation coefficient (r) is marked with asterisks (** = significance at $p \leq 0.001$).

and total phenols was linear with a coefficient of determination $R^2 = 0.8683$, which assumes a strong relationship between the observed parameters. Table 3 shows the results of the correlation coefficients (r) between the total phenol content (mg l⁻¹) and antioxidant activity (mmol TE l⁻¹) in the aqueous samples of lemon balm leaves extracted with the classical method and with ultrasound. A high correlation coefficient ($p \leq 0.0001$) was observed between the total phenol content ($r = 0.932$), non-flavonoids ($r = 0.938$), and antioxidant activity of lemon balm aqueous leaf extracts.

Table 4 presents the results of the total phenolic, flavonoid, and non-flavonoid content and antioxidant activity of fresh peppermint leaves and aqueous leaf samples extracted classically and with ultrasound. A high significant statistical difference ($p \leq 0.0001$) was observed for all the analysed phenol compounds (total phenols, flavonoids, and non-flavonoids) depending on the extraction technique. In the samples of fresh peppermint leaves, a high content of total phenols (1 722.25 mg l⁻¹) was determined, which is a slightly lower value, compared with the total phenol content of fresh lemon balm leaves. The results obtained are consistent with other literature data, which emphasize a significantly high phenol content of peppermint leaves (Lv *et al.*, 2012; Kapp *et al.*, 2013; Riachi and De Maria, 2015). During the 24 h of classical extraction of fresh peppermint leaves in water, the total phenol content was 1 472.49 mg l⁻¹, which is an about 15% lower value in comparison with the total phenol content identified in fresh leaves. Based on the data above mentioned, it should be emphasized that the applied classical extraction method significantly contributed to the separation of phenolic compounds. During the ultrasonic extraction of the aqueous peppermint samples, an about 60% increase in the total phenol content in the period from 5 to 25 min was determined. The lower total phenol content was determined during ultrasonic extraction in a maximum applied time of 30 min. An increasing trend of the total flavonoid and non-flavonoid content during ultrasonic extraction for

T a b l e 4. Total phenol, non-flavonoid, and flavonoid content and antioxidant capacity of leaves and aqueous samples of peppermint leaves extracted with the ultrasound and classical methods

Extraction technique	Total phenols	Non-flavonoids	Flavonoids	Antioxidant capacity
	(mg l⁻¹ extract)			(mmol TE l⁻¹)
Leaves	1722.25±2.62 a	1132.96±0.99 a	589.29±3.61 a	2.30±0.375 a
Classical extraction	1472.49±0.765 b	1080.76±1.28 b	391.73±0.515 b	2.15±5.12 b
UES1	332.45±1.595 h	200.91±0.705 h	131.54±2.3 h	1.98±0.62 d
UES2	355.57±0.575 g	213.81±1.345 g	141.76±0.77 g	2.02±18,87 cd
UES3	399.31±0.01 f	222.81±1.15 f	176.50±1.15 f	2.03±8.1 cd
UES4	449.45±0.705 e	263.29±0.755 d	186.15±1.47 e	2.05±1.645 cd
UES5	534.07±0.125 c	288.08±0.01 c	245.99±0.125 c	2.08±1.56 bc
UES6	467.55±0.955 d	257.77±1.15 e	209.78±0.195 d	2.07±1.405 bcd

Explanations as in Table 2.

a period from 5 to 25 min was recorded. The antioxidant activity of fresh peppermint leaves was lower than the antioxidant values determined for fresh lemon balm leaves, which was expected, since fresh lemon balm leaves had higher total phenol content. Oh *et al.* (2013) and Riachi and De Maria (2015) emphasize the very high antioxidant capacity of aqueous peppermint solutions, comparing different methods for testing the antioxidant activity (FRAP, ORAC, ABTS, DPPH).

A strong correlation between total phenolic compounds (flavonoids and non-flavonoids) of peppermint samples (extracted classically and with ultrasound) and antioxidant activity was determined. The linear regression between antioxidant activity measured with the ABTS method and the total phenol content of the peppermint aqueous extracts

are presented in Fig. 1b. The total phenol content of peppermint aqueous extracts was found to correlate significantly with the antioxidant capacity (ABTS). The relationship between antioxidant capacity and total phenolic compounds was linear with a coefficient of determination $R^2=0.7628$, which assumes a strong relationship between the observed parameters. A high correlation coefficient ($p \leq 0.0001$) was observed between the total flavonoids ($r=0.961$), total phenol content ($r=0.874$), non-flavonoids ($r=0.836$ with $p \leq 0.001$) and antioxidant activity of peppermint aqueous leaf extracts (Table 3).

The total chlorophyll, chlorophyll a, chlorophyll b, and carotenoid content of fresh lemon balm and peppermint leaves and their aqueous samples extracted classically and ultrasonic are shown in Tables 5 and 6. Lemon balm

T a b l e 5. Total chlorophylls, chlorophyll a, chlorophyll b, and total carotenoids in leaves and aqueous samples of lemon balm leaves extracted with the ultrasound and classical methods

Extraction technique	Total chlorophylls	Chlorophyll a	Chlorophyll b	Total carotenoids
	(mg g^{-1})			
Leaves	177.21±1.92 a	115.57±1.16 a	61.64±0.76 a	56.09±0.22 a
Classical extraction	2.79±0.94 d	0.83±0.29 cd	1.96±0.64 de	3.83±0.85 b
UES1	0.93±0.01 e	0.31±0.01 d	0.62±0.01 f	0.56±0.004 g
UES2	2.24±0.04 de	0.74±0.015 cd	1.49±0.03 e	0.79±0.004 f
UES3	3.01±0.11 d	0.99±0.04 cd	2.02±0.07 de	1.03±0.02 e
UES4	6.39±0.02 b	2.16±0.007 b	4.23±0.013 b	3.85±0.02 b
UES5	4.64±0.09 c	1.55±0.04 bc	3.09±0.05 c	3.01±0.03 c
UES6	3.24±0.06 cd	1.06±0.01 cd	2.19±0.045 d	2.35±0.004 d

Explanations as in Table 2.

T a b l e 6. Total chlorophylls, chlorophyll a, chlorophyll b, and total carotenoids of leaves and aqueous samples of peppermint leaves extracted with the ultrasound and classical methods

Extraction technique	Total chlorophylls	Chlorophyll a	Chlorophyll b	Total carotenoids
	(mg g^{-1})			
Leaves	703.15±6.67 a	454.48±4.04 a	248.66±2.63 a	163.43±1.53 a
Classical extraction	4.37±0.64 f	1.33±0.23 h	3.04±0.41 f	6.66±0.21 b
UES1	4.10±0.001 g	1.45±0.02 g	2.58±0.04 g	2.72±0.015 f
UES2	5.04±0.04 e	1.83±0.006 e	3.21±0.03 e	3.06±0.017 e
UES3	5.39±0.001 d	1.96±0.001 d	3.43±0.001 d	3.32±0.011 d
UES4	9.84±0.102 b	3.55±0.03 b	6.29±0.07 b	5.49±0.04 c
UES5	6.77±0.123 c	2.40±0.04 c	4.37±0.08 c	3.28±0.04 d
UES6	4.52±0.06 f	1.59±0.02 f	2.93±0.04 f	1.09±0.001 g

Explanations as in Table 2.

and peppermint are characterized by high content of green pigments, chlorophylls, which is confirmed by numerous scientific investigations (Grzeszczuk and Jadczak, 2009; Loranty *et al.*, 2010; Komes *et al.*, 2011). According to the result of this research, fresh lemon balm leaves contained on average 177.21, 115.57, and 61.64 mg g^{-1} of total chlorophylls, chlorophyll a, and chlorophyll b; while fresh peppermint leaves exhibited significantly higher amounts: 703.15, 454.48, and 248.66 mg g^{-1} of total chlorophylls, chlorophyll a, and chlorophyll b, respectively. Fresh lemon balm and peppermint leaves also contained significant amounts of total carotenoids: lemon balm 56.09 mg g^{-1} and peppermint even three times higher *ie* 163.43 mg g^{-1}. The extraction technique significantly affected the total chlorophyll (chlorophyll a and chlorophyll b) and carotenoid content in lemon balm and peppermint aqueous samples with p value less than 0.0001. During the ultrasonic extraction of lemon balm leaves for 20 min (UES4), a significant increase in the total chlorophyll content was determined, even 56% higher compared with the samples extracted classically. Also, the same increasing trend during ultrasonic extraction for 20 min was determined for the chlorophyll a and chlorophyll b content. The chlorophyll a content was even 62%, and chlorophyll b 54% higher compared to the samples extracted classically. In the period from 25 (UES5) to 30 min (UES6) of ultrasonic extraction, a significant decrease in the total chlorophyll (chlorophyll a and b) content was determined, but the final value was still higher than the value obtained in the sample extracted classically. Numerous literature data emphasize the extreme effectiveness and significant yields in the application of the ultrasound techniques in the extraction of chlorophylls and its derivatives from various plant materials (Barba *et al.*, 2015b; Grimi *et al.*, 2014; Roselló-Soto *et al.*, 2015a, 2015b). In their research, Kong *et al.* (2014) emphasize a significant yield increase (59%) in total chlorophyll content recovered from a *Chlorella vulgaris* residue by ultrasound application compared to conventional heat

extraction. The total carotenoid content determined in fresh lemon balm leaves was 56.09 mg g^{-1}, which is a significantly lower value than the value determined for the carotenoid content in fresh peppermint leaves (163.43 mg g^{-1}). Also, the carotenoid content significantly changed depending on the applied extraction technique in the samples of lemon balm and peppermint leaves. The highest total carotenoid content was determined for the aqueous samples of lemon balm leaves extracted with ultrasound (3.83 mg g^{-1}), while the highest content 6.66 mg g^{-1} in the aqueous samples of peppermint leaves was determined by applying the classical extraction. During the ultrasonic extraction of the lemon balm samples in the period from 5 to 20 min, an increase in the total carotenoid content from 0.56 (UES1) to 3.85 mg g^{-1} (UES4) was determined, which is an increase by approximately 85%. In the period of 25(UES5) and 30 min (UES6) of ultrasonic extraction, a decrease in the total carotenoid content was determined. At the ultrasonic extraction of the aqueous peppermint leaf samples, an increase in the total carotenoid content in the period from 5 to 20 min was determined, with the highest value of 5.49 mg g^{-1} (UES4), which is an increase by about 51%. The increasing trend for the total carotenoid content is in line with other literature data, which emphasize the extreme effectiveness of ultrasound in carotenoid extraction from various plant materials such as carrots, algae, tomatoes etc. in comparison with the classical techniques (Kumcuoglu *et al.*, 2014; Roselló-Soto *et al.*, 2015b).

Figures 2 and 3 show linear regression between the total chlorophyll and total carotenoid contents (mg g^{-1}), and antioxidant activity (mmol TE l^{-1}) measured in different aqueous extracts of lemon balm and peppermint leaves. Linear regression analysis between the total chlorophyll content and antioxidant activity of aqueous lemon balm and peppermint samples did not show a significant correlation (Fig. 2), which was also determined by analysing the correlation coefficients (Table 3). The results obtained differ from other literature data. Namely, chlorophylls are chemical
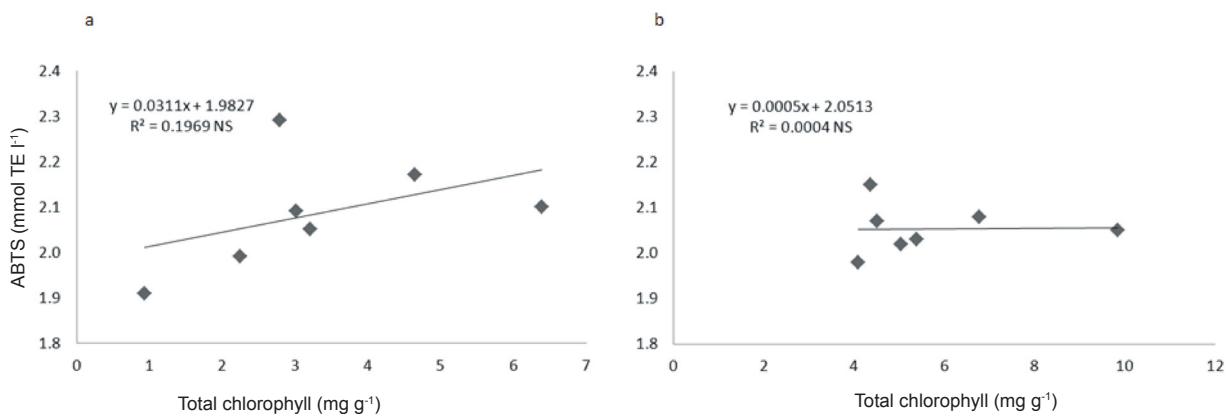

Fig. 2. Linear regression between the total chlorophyll content (mg g^{-1}) and antioxidant activity (mmol TE l^{-1}) in the aqueous extracts of: a – lemon balm and b – peppermint leaf samples. Significance of the correlation coefficient (r) is marked with asterisks, ***significance at p≤0.0001.
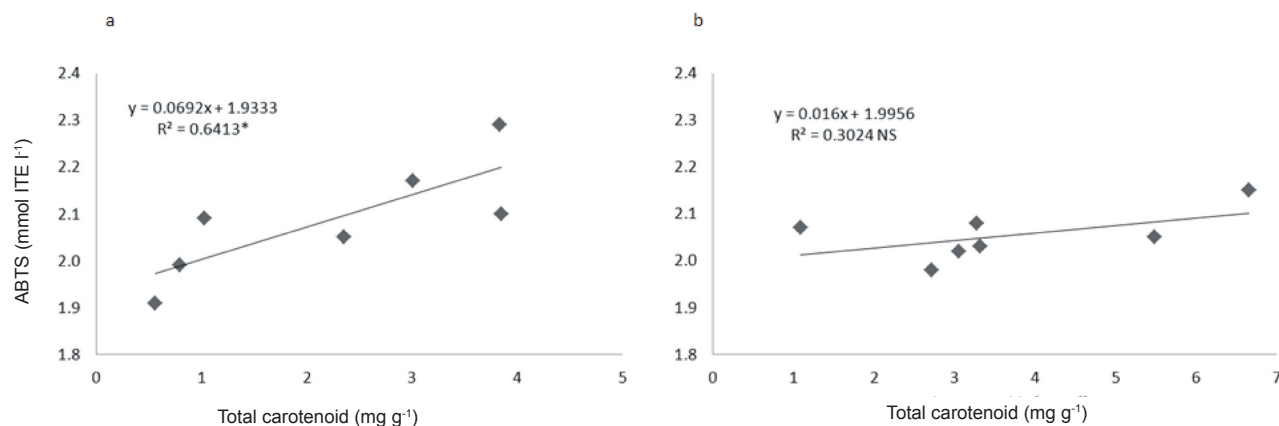
Fig. 3. Linear regression between the total carotenoid content (mg g⁻¹) and antioxidant activity (mmol TE l⁻¹) in the aqueous extracts of: a – lemon balm and b – peppermint leaf samples. Explanations as in Fig. 2.

compounds with a proven significant antioxidant effect, due to which they show a positive effect on human health (Roselló-Soto *et al.*, 2015b). The total carotenoid content of the lemon balm aqueous extracts significantly correlate $(0.01<p<0.05)$ with the antioxidant activity (ABTS) at a coefficient of determination $R^2=0.6413$ (Fig. 3a). Linear regression analysis between the total carotenoid content and antioxidant activity of the aqueous peppermint samples did not show a significant correlation with the determined coefficient $R^2=0.3024$ (Fig. 3b) and correlation coefficient $r=0.549$ (Table 3). Carotenoids and their derivatives are compounds that exhibit strong antioxidant activity (Mueller and Boehm, 2011; Roselló-Soto *et al.*, 2015b). In this research, the extraction method is the main reason why no significant correlation between the total chlorophyll, carotenoid content, and antioxidant activity was determined.

CONCLUSIONS

1. The extraction technique significantly affected the content of all the investigated bioactive compounds of the lemon balm and peppermint aqueous extracts.

2. With the conventional extraction method, higher contents of total phenolic compounds and antioxidant activity were determined, in comparison with ultrasonic extraction.

3. Ultrasonic extraction for 25 min yielded a significant total phenol content and antioxidant activity.

4. By ultrasonic extraction for 20 min, higher values of total chlorophylls (chlorophyll a and b) and carotenoids were obtained, in comparison with the conventional method.

5. Application of ultrasound significantly shortens the extraction period compared with the conventional method in which the extract time amounts to 24 h.

6. During ultrasonic extraction for 20 and 25 min, high values of total phenolic compounds, total chlorophylls, carotenoids, and antioxidant activity were obtained.

7. A significant, positive correlation between the total phenol content and antioxidant activity of the aqueous lemon balm and peppermint extracts was determined.

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