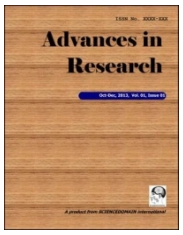

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Quantification of Total Flavonoids and Phenolic Acids from Microwave Irradiated and Non-irradiated Plants

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Authors' contributions

Author IL together with the Author MLS managed the analyses of the extracts. Author MS managed the literature searches and extracts the bioactive compounds from plants and Authors DP performed the statistical analysis and supervised the growth of plants. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The aim of the present study was to investigate the effect of the low power microwave radiations on the concentration of phenolic compounds (flavonoids and phenolic acids) from *Satureja hortensis* L. and *Ocimum basilicum* L., after it was established the efficient method of extraction.
Study Design: The extracts were obtained from irradiated and non-irradiated plants, were analysed spectrophotometrically and were drawn the conclusions.
Place and Duration of Study: The experiments were performed in the Physics of Nanostructured Systems Department, National Institute for Research and Development of Isotopic and Molecular Technologies and Department of Molecular Biology, Faculty of Biology and Geology, Babeş-Bolyai University between June 2012 and June 2013.
Methodology: Experiment was carried out by exposing the plants to microwave radiation modulated by a specific WLAN communications protocol (2.4...2.49 GHz with intensity of 70 mW m^{-2}). The test plants and the control plants were subjected to the same environment during two weeks.
Results: The data obtained for the plants subjected to microwave treatments were analysed and compared with those registered for the non-irradiated plants. Results

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showed that the concentration of total flavonoids in both studied species is significantly higher in the microwave irradiated compared to the non-irradiated plants.
Conclusion: This paper proposes to investigate the effect of microwave on the concentration of phenolic compounds from plants. The results showed that the concentration of total flavonoids and total phenolic acids is higher in irradiated plants compared to the non-irradiated plants. The amount of flavonoids, respectively phenolic acids in the irradiated *Satureja hortensis* L. is 35.7%, respectively of 0.89% higher than in the non-irradiated plant, while in the irradiated *Ocimum basilicum* L. is of 24.3%, respectively of 11.59% higher than in non-irradiated plant.

Keywords: Flavonoids; phenolic acids; microwave irradiation; spectrophotometry; extraction.

1. INTRODUCTION

Constant concern for the quality of life and the use of the advanced industrial technologies has determined, by end of the 20th century, an increase in the number of sources of non-ionizing electromagnetic radiation from the microwave domain. These sources include radio and TV emitters, microwave ovens, telephone receivers from the cellular telephone systems, radar installations as well as various equipments used in industry, medicine, commerce. These sources can cause damage depending on the power level, frequency, duration of exposure, type of wave: pulsed or continuous and the properties of exposed tissue [1].

Plants are the main provider of phytochemical compounds used in different industrial branches such as the pharmaceutical products, foods, cosmetics, agrochemicals. Therefore, it is beneficial to investigate their interaction with today's increased exposure to microwave frequency field. Large classes of chemicals which are found in plants are phenolic compounds that have attracted much attention in the last decades due to their properties and the hope that they will show beneficial health effects, when taken as a dietary input or as complement [2]. Phenolic acids constitute one of the most numerous and widely distributed groups of phytochemicals in the plant kingdom [3]. Phenolic acids account for approximately one-third of the total dietary intake of phenolic compounds, while flavonoids account for the remaining two-thirds [4].

Plant phenolic compounds can be found in different forms, thus, their extraction from the plant material raises particular difficulties [5, 6]. Most of the current analysis protocols use classic extraction techniques, among which maceration, Soxhlet and refluxing extractions are the most commonly used. Due to the disadvantages these techniques present, there is a tendency of replacing them with other techniques that require less reagents, energy and time, while exhibiting similar efficiency. Consequently, techniques such as ultrasound-assisted extraction (UAE) [7,8], microwave-assisted extraction (MAE) [9] and accelerated solvent extraction (ASE) [10,11] have been implemented.

Plant extracts can be analysed both globally, therefore non-specifically by determining the total content of phenolic compounds, as well as specifically. Therefore, non-specifically, the phenolic compounds can be determined spectrophotometrically using a specific colour reagent. Specific determination (identification and quantification) can be performed by chromatographic methods; the most widely used being the high-performance liquid

chromatography coupled with photodiode array detector (HPLC-PDA) or coupled with a mass spectrometer (HPLC-MS).

Savory (*Satureja hortensis* L.) and basil (*Ocimum basilicum* L.) are two aromatic herbs in the *Lamiaceae* family, known for their culinary and therapeutic properties [12-17]. Therefore, the influence of low power microwave field on the content of total flavonoids and total phenolic acids in the two plants is of commercial interest.

To date, most of the published research has focused on the effects that microwave radiations have on seed germination, growth rate of seedling and content of chlorophylls and carotenoids, while studies on the influence of microwave on the content of phenolic compounds and essential oils are scarce [1,8-20].

The aim of the current research was to establish an efficient method for the extraction of flavonoids and phenolic acids from plants and to use the method to determine the influence of microwave irradiation on the level of these compounds in two aromatic herbs.

2. MATERIALS AND METHODS

2.1 Apparatus

Spectrophotometric measurements were performed with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan) with 1 cm optical path length quartz cuvette. An ultrasound bath (Elmasonic S 15H, 37 kHz) and a home-made microwave apparatus were used for the extraction of bioactive compounds. Rotary evaporator Laborota 4011 (Heildolph, Germany) was used for evaporating to dryness the obtained extracts.

2.2 Reagents

Ethanol used for the extraction of bioactive compounds was purchased from Chimopar, Romania. Chemicals used for spectrophotometric analysis: sodium acetate, sodium hydroxide, sodium nitrite, aluminium chloride and sodium molybdate were purchased from Sigma-Aldrich, Germany, while hydrochloric acid from Microchim, Romania. Caffeic acid and rutin standards were employed from Sigma-Aldrich, Germany. All chemicals were of analytical grade.

2.3 Plant Material

Plant species used for the current experiment were savory - *Satureja hortensis* L. and basil - *Ocimum basilicum* L.

To establish the most efficient method for extraction of flavonoids and phenolic acids from plants, dried savory and basil plant material were commercially purchased from Kotanyi GmbH.

In the experiments where the effects of the microwave irradiation on the bioactive compounds of plants were assessed, savory and basil were primarily grown from seeds. Seeds from ARO (Romania) were sown in circular pots with 200 g of soil. Once the seeds emerged, the seedlings were re-picked leaving one plant per pot. At three weeks after

sowing, plants were placed in two identical anechoic chambers [21]. Test (irradiated) and control (non-irradiated) plants were subjected to the same environment.

Stimulation was performed with microwave radiation modulated by a specific WLAN communications protocol, in the 2.4...2.49 GHz frequency band, at a power density in the plants of 70 mW m^{-2}. The irradiation was performed over two weeks, after which plants were removed from the chambers and analysed.

Fresh leaves were excised manually from plants and then stored in the dark, at room temperature (~25ºC) until completely dried.

2.4 Extraction Procedure

After grinding with a hand mill (grinder), the powder was weighed in portions of 0.5 g and subjected to solvent extraction with ethanol-water using various techniques. The solvent extraction was performed using the following ethanol-water mixtures: pure ethanol; ethanol-water (90:10, v/v); ethanol-water (80:20, v/v); ethanol-water (70:30, v/v); ethanol-water (60:40, v/v); ethanol-water (50:50, v/v) and ethanol-water (40:60, v/v).

Four extraction methods of flavonoids and phenolic acids from *Satureja hortensis* L. and *Ocimum basilicum* L. were investigated and compared, including microwave-assisted extraction, ultrasonic-assisted extraction, refluxing and maceration. For establishing the most efficient extraction method of flavonoids and phenolic acids, the plant material of *Satureja hortensis* L. and *Ocimum basilicum* L. were commercially purchased.

Maceration was performed 14 days at room temperature with 40 mL of extraction solvents (ethanol-water mixtures).

Refluxing was performed after 30 min soaking for 30 min with 40 mL of extraction solvents (ethanol-water mixtures).

Ultrasound-assisted extraction (UAE) was performed using an Elmasonic S bath at 37 kHz and an installed power of 95 W. The sample was soaked 30 min with 40 mL extraction solvent (ethanol-water mixtures) and then sonicated for 30 min at 70°C.

Microwave-assisted extraction (MAE) was performed using a home-made apparatus [22] which allows the control of the time, temperature and duty cycle during extraction. The features of this apparatus: microwaves power at 2.45 GHz frequency, programable power between 100 W - 900 W; control of microwaves energy by the treatment time (programable activation in pulses or continuos mode); activation in power pulses with the repetition frequency of 1 Hz, programable pulse width in the range of 100 ms – 1 s; stand alone control by microcontroller RISC with LCD display of the control parameters; power supply 220 V / 50 Hz, max.1200 VA; treatment on single mono-modale cavities having volumes between (6 mL – 100 mL).

The plant material (0.5 g) together with extraction solvent (40 mL) was placed into the extraction cell. The sample was soaked 30 minutes followed by microwave extraction. Taking in account the specificity of plant material the following parameter were selected: maximum temperature 70ºC, action time 2 min and duty cycle of 40% at an installed power of 900 W.

Each extraction was performed in three parallel samples. After filtration and washing, the extracts were evaporated to dryness in a rotary evaporator and the residues were re-dissolved in 10 mL extraction solvent.

2.5 Determination of Total Flavonoids Contents in the Plant Extracts

The concentration of total flavonoids in the plant extracts was determined by the Romanian Pharmacopoeia method [23]. The concentration of total flavonoids was determined using rutin as reference compound: 1 mL of plant extract was mixed with 5.0 mL of sodium acetate 100 g L^{-1}, 3.0 mL of aluminium chloride 25 g L^{-1}, and filled up to 25 mL by water in a calibrated flask. The absorbance was recorded at 430 nm after 15 min. The blank sample was prepared from 1 mL of plant extract, 5.0 mL of sodium acetate 100 g L^{-1} and water up to 25 mL. The absorbance of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from a 1 mg mL^{-1} stock solution with concentration ranges between 0.02-0.4 mg mL^{-1}. The concentration of total flavonoids was determined using the equation obtained from the calibration curve of rutin graph ($y=1.2344x+0.0071$; $R^2=0.9986$).

2.6 Determination of Total Phenolic Acids Contents in the Plant Extracts

The content of total phenolic acids in the plant material was determined using the spectrophotometric method with Arnow's reagent according to the procedure described in the Romanian Pharmacopoeia X [23]. The content of the total phenolic acids was determined using caffeic acid as reference compound: 1 mL of plant extract was mixed with 1 mL of hydrochloric acid 0.5 N, 1 mL of Arnow's reagent, 1 mL of sodium hydroxide 1 N and filled up to 10 mL by water in a calibrated flask. The absorbance was determined at 500 nm. A blank sample was also prepared from 1 mL of plant extract, 1 mL of hydrochloric acid 0.5 N, 1 mL of sodium hydroxide 1 N and water up to 25 mL. Standard caffeic acid solutions were prepared from a 1 mg mL^{-1} stock solution with concentration ranges between 0.005-0.2 mg mL^{-1}. The content of total phenolic acids, expressed as caffeic acid equivalent on dry weight, was calculated based on the equation obtained from the calibration curve of caffeic acid ($y=1.7324x+0.0216$; $R^2=0.9994$).

2.7 Statistical Analysis

All experimental measurements were carried out in triplicate and were expressed as the mean of three analyses ± standard deviation. Standard deviations, the regression equations and correlation coefficient (R^2) were obtained using Microsoft Excel 2003 software data management system. One way analysis of variance (ANOVA) and Tukey tests were used to confirm differences between means ($P < 0.001$).

3. RESULTS AND DISCUSSION

Preliminary experiments were conducted to determine the most effective conditions for obtaining the phenolic compound extracts from the microwave irradiated and non-irradiated plants. Therefore, the ratio of ethanol-water volumes in the extraction mixtures and the extraction techniques were varied. Quantification of flavonoids was performed by means of UV–Vis spectrophotometry with aluminium chloride as colour reagent [23]. The content of total phenolic acids in the plant material was spectrophotometrically determined using the Arnow's reagent [23].

The extracts obtained with various ratios of ethanol-water were compared for all extractions techniques (Fig. 1 and Fig. 2).

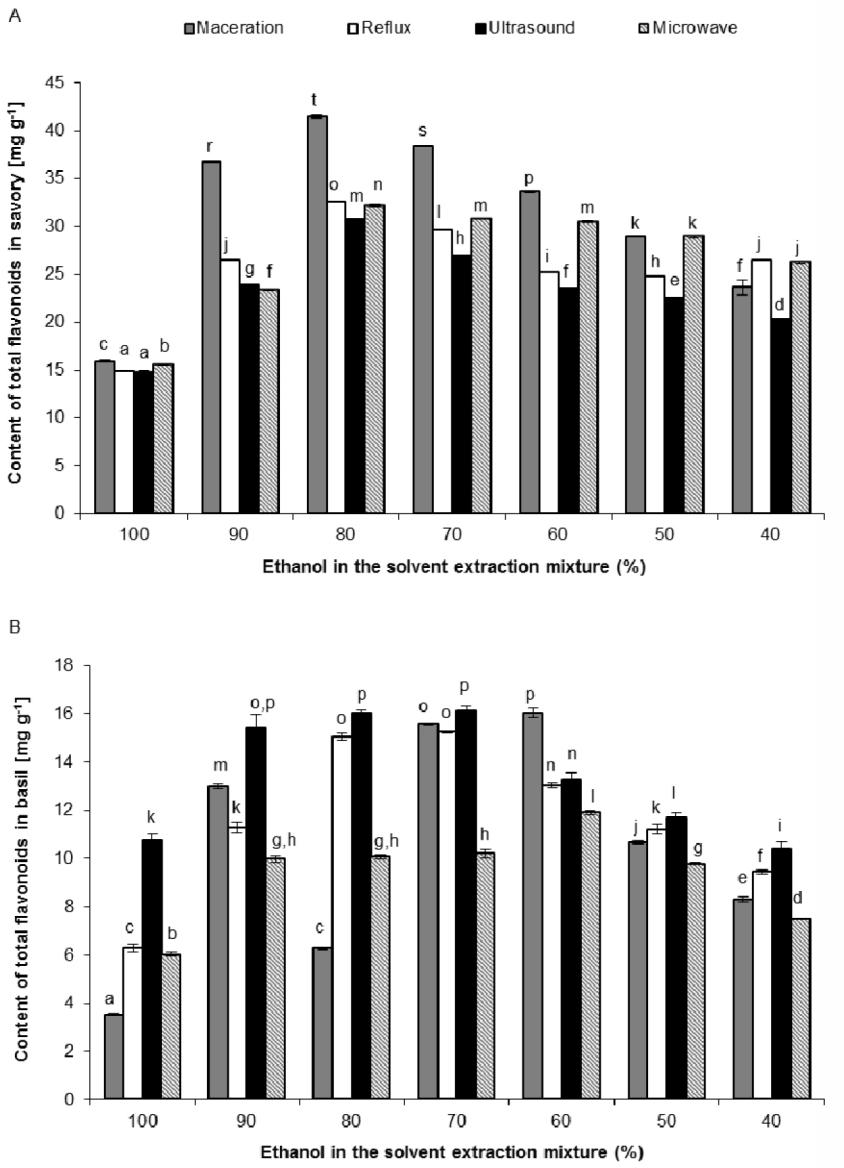

Fig. 1. Comparison of the content of total flavonoids in savory (A) and basil - (B) leaves depending on the technique and the percentage of ethanol in the extraction mixture used (mean ± SD, n = 3). Bars with different letters are significantly different (confirmed by Tukey test).

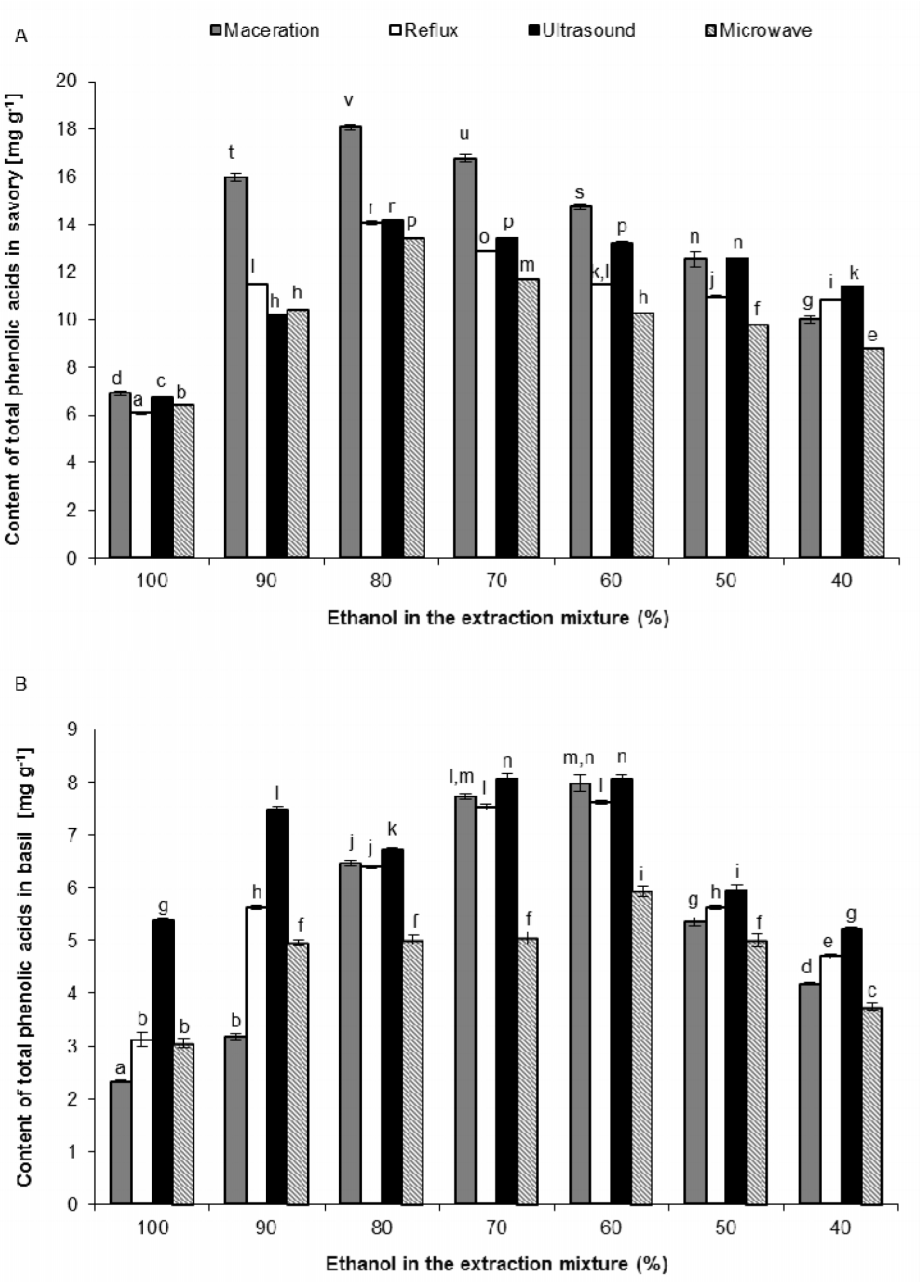

Fig. 2. Comparison of concentration of phenolic acids in savory (A) and basil (B) leaves depending on the technique and the percentage of ethanol in the extraction mixture used (mean ± SD, n = 3). Bars with different letters are significantly different (confirmed by Tukey test).

Comparing the UV-VIS spectra, similar results were obtained for flavonoids and phenolic acids. From quantitative determination, it was concluded that the effective extraction

technique depends on the type of plant. Maceration, followed by ultrasound-assisted extraction was found to be the most efficient extraction technique for savory (Fig. 1A and Fig. 2A). Regardless of the extraction method used, the 80% (v/v) of ethanol in the extraction mixture has shown to be the most efficient (Fig. 1A and Fig. 2A). For basil, the UAE using 60% (v/v) of ethanol in the extraction mixture was proven to be the most efficient technique (Fig. 1B and Fig. 2B). Since maceration requires a long time (14 days), the UAE (30 min) was chosen as the extraction method used for both plants.

Therefore, flavonoids and phenolic acids from non-irradiated and microwave irradiated plants were extracted by ultrasound-assisted extraction. Extraction was performed at 70°C for 30 minutes. The results obtained from the plants subjected to microwave treatments were analysed and compared with the data registered for the non-irradiated plants (Table 1).

Table 1. The content of total flavonoids and phenolic acids in extracts (mg g^{-1}) from non-irradiated and microwave irradiated plants

Plant	Treatment	Phenolic compounds content[1]	
		Flavonoids[2] (mg g^{-1})	Phenolic acids[3] (mg g^{-1})
Savory	Irradiated	5.893±0.007	2.709±0.002
	Non-irradiated	4.334±0.014	2.685±0.007
Basil	Irradiated	4.748±0.005	2.894±0.005
	Non-irradiated	3.819±0.005	2.592±0.001

[1]Results are presented as mean ± SD (n=3).
[2]Content calculated as rutin equivalents.
[3]Content calculated as caffeic acid equivalents.

Results showed that the concentration of total flavonoids in both studied species is significantly higher in the microwave irradiated compared to the non-irradiated plants, being 36% and 24.3% higher in savory and basil, respectively. The concentration of phenolic acids (caffeic acid type) was 11.7% significantly higher in the microwave irradiated compared to non-irradiated plants only for basil. In the case of irradiated savory, the increase of the phenolic acids concentration is not significantly higher compared to the non-irradiated plant.

4. CONCLUSION

UAE using a mixture of ethanol-water (80:20, v/v) in the case of savory and ethanol-water (60:40, v/v) in the case of basil has proved to be an efficient extraction method for phenolic compounds (flavonoids and phenolic acids). These extraction conditions were used further for the extraction of phenolic compounds from microwave irradiated and non-irradiated plants. The presence of the microwaves has influence on the composition of bioactive compounds followed in this study. The total amount of flavonoids and phenolic acids (caffeic acid type) is higher in irradiated than in the non-irradiated plants. Therefore, the amount of flavonoids, respectively phenolic acids in the irradiated savory is 35.97%, respectively 0.89% higher than in the non-irradiated plant, while in the irradiated basil is 24.3%, respectively 11.65% higher than in the non-irradiated plant.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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