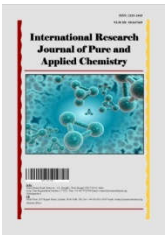

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Antioxidant Capacity and Total Phenolic and Flavonoid Contents of Methanolic Extracts of *Urtica dioica L.* by Different Extraction Techniques

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SB, EH, HA and AZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EB, SS, ECK and MI performed the analyses of the study. Authors EK and MS performed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

In this study, the efficacy of different extraction techniques (maceration, ultrasound-assisted and Soxhlet extraction) on the content of biologically active components in extracts from fresh and dried nettle leaves, and their antioxidant activity were analyzed. Methanol was used as the solvent. Total phenolic content and antioxidant capacity were determined by Folin-Ciocalteu, DPPH and FRAP methods, respectively. High content of total phenolic compounds and high antioxidant activity were recorded in extracts of dried nettle. Extracts obtained from fresh nettle samples showed significantly lower content of analyzed bioactive components and lower antioxidant activity. In the case of all extracts, Soxhlet extraction proved to be the most efficient, and maceration the least efficient extraction technique for isolation of bioactive components from nettle leaves.

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1. INTRODUCTION

Urtica dioica (Urticaceae), commonly known as nettle is an herbaceous perennial species. Growing up to 1 m high, nettle produces erect and wiry stems that hold up its opposite, roughly textured, serrated leaves [1-4]. The plant is rich of chemical component and composition [5], which is why it has a long history of use as a food, medicine and against hair loss [1,2,6]. Nettle contains neuromodulators acetylcholine, histamine, serotonin, which are also neurotransmitters, and choline which is the precursor for acetylcholine. The nettle leaves contain a significant number of biologically active compounds, such as terpenoids, carotenoids including β-carotene, neoxanthin, violaxanthin, lutein and lycopene, fatty acids, especially palmitic, cis-9,12-linoleic and α-linolenic acids, different polyphenolic compounds, essential amino acids, chlorophyll, vitamins such as A, B, C, E and K, tannins, carbohydrates, sterols, polysaccharides, isolectins and minerals [7-19]. Scientific studies have shown that *Urtica dioica* extracts have antimicrobial, anti-inflammatory, antidiabetic, and anti-aging effects, which is associated with the content of phenolic compounds in them [20-22]. Phenolic compounds are famous group of secondary metabolites with wide pharmacological activities [23]. The term „plant phenolics" encompasses simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, up to hydrolysable and condensed tannins, lignans, and lignins [24]. It is generally accepted that therapeutic effects of many plant species are attributed to the presence of antioxidative phenolics in their tissues [25], which is why numerous studies have been published on their extraction from various plant species and the determination of their content and antioxidant properties. Extraction of phenolics from plant materials is carried out by various conventional and advanced techniques, such as maceration, Soxhlet, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, etc. and among them the most widely used techniques employ solvents [26]. Solvent extraction can be defined as a process of separation by applying a solvent to extract the targeted component (solute) from the solid [27]. Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water [28]. Many published studies of *Urtica dioica* have examined and compared the antioxidant activity and other health benefits of nettle extracts, using different solvents. Nettle extract obtained using ethyl acetate showed maximum antioxidant activity compared to those obtained with petroleum ether, n-butanol and ethanol [29]. Ethyl acetate extracts also showed obvious antibacterial and antioxidant activities in comparison to dandelion ethyl acetate extracts [30]. Hydroalcoholic extract of *Urtica dioica* showed positive in-vitro antioxidant activity and nettle was described as natural antioxidant that can replace the synthetic ones to be used in foods and cosmetics [31]. In studies on aqueous extracts of nettle biological compounds, antioxidant capacity and their anti-diabetic, antimutagenic and hypotensive effects were determined [9,21,32,33]. Methanolic extracts studies suggested that *Urtica dioica* has a protective capacity and antioxidant activity against cisplatin toxicity in EAT-bearing mice and can efficiently disslove calcium oxalate renal stones in male Sprague-Dawley rats [34,35]. In this paper, the influence of the applied extraction technique on total phenolic and flavonoid contents in extracts obtained from fresh and dried *Urtica dioica* leaves was investigated, where methanol was used as a solvent.Given the potential benefits of nettle bioactive compounds as antioxidants, it is useful to have more studies available on the effect of the type of extraction method on their amount in extracts, for *Urtica dioica* of different geographical origin and seasonal harvest conditions, and in regard,this paper represents a significant contribution.

2. MATERIALS AND METHODS

All chemicals used were of analytical grade and were used as received, without any further purification.Folin-Ciocalteu reagent and sodium carbonate purchased from Merck (Darmstadt, Germany). The 2,2-diphenyl-1-picrylhydrazyl, sodiumacetate and gallic acid, ferric chloride and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma-Aldrich (St. Louis, USA). Methanol was purchased from Semikem (Sarajevo, B&H).

2.1 Sampling and Preparation of Materials for Analysis

Urtica dioica, which has been harvested in Tuzla area (Bosnia and Herzegovina) in June, was cleaned, and leaves were separated from the

rest of the plant. One part of the fresh leaves was immediately subjected to extraction, and the other part was previously dried for seven days at room temperature, in a dark and dry place. Prior to the extraction procedure, fresh nettle samples were chopped with a knife, and dry samples grounded in an electric mill.

2.2 Preparation of Methanol Extracts

Three methods were used for extraction: Soxhlet extraction, ultrasound-assisted extraction and maceration. In all three cases, 10 grams of fresh or dried nettle were weighed and transferred to a flat-bottomed balloon, or paper tube (in the case of Soxhlet extraction), and poured with 150 mL of methanol. Ultrasound assisted extraction was performed in an Elmasonic S ultrasonic bath, without heating. Maceration was performed at room temperature with stirring at 300 rpm with Tehtnica Vibromix 40. After four hours of extraction for all three methods, the extracts were filtered through filter paper and then stored in a dark and cool place before analysis.

2.3 Determination of Total Phenolic Content (TPC)

Total phenolic compounds present in the extracts were quantified spectrophotometrically through the Folin-Ciocalteu test following the protocol [36], with some modifications. 200 µL of extract was mixed with 2.54 mL of 10% Folin-Ciocalteu reagent. After 5 min, 420 µL of 10% sodium carbonate was added. 910 µL of distilled water was added to each sample prior to measuring. The absorbance of the resulting blue-coloured solution was measured at 765 nm. Quantitative measurements were performed, based on a standard calibration curve of gallic acid ($y = 0{,}0042x + 0{,}0076$, $R^2 = 0{,}9998$). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrammes per 100 grams of nettle sample.

2.4 Determination of Total Flavonoid Content (TFC)

Total flavonoid content in the extracts was determined by the previously described method [37], with some modifications. 1 mL of extract solution was mixed with 0.3 mL of 5% sodium nitrite. 0.3 mL of 10% aluminium chloride was added after 5 minutes. After 6 minutes of incubation at room temperature, 1 mL of 1 M sodium hydroxide was added to the reaction mixture, and the final volume was made up to 10 mL with distilled water. Absorbance of sample was measured against the blank at 510 nm using a spectrophotometer. The results were derived from the calibration curve ($y = 3{,}024x - 0{,}0034$; $R^2 = 0{,}9984$) of quercetin and expressed in quercetin equivalents (QE) per 100 grams of nettle sample.

2.5 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power of each extract, which reflects the antioxidant activity, was determined following the protocol [38]. 3 mL of prepared FRAP reagent was mixed with 100 µL of diluted extracts. Absorbance at 593 nm was recorded after a 30 min incubation at 37°C. The FRAP value was calculated from the calibration curve of iron (II) sulfate heptahydrate ($y = 0{,}001x + 0{,}0698$; $R^2 = 0{,}9997$).

2.6 DPPH Radical Scavenging Activity

2,2-diphenyl-1-picryl-hydrazyl (DPPH) method was performed according to the previously described method [39]. A series of solutions in test tubes was made by adding different volumes of extract supplemented with up to 2 mL of methanol. 0.5 mL of 0.5 mM DPPH solution was added and the samples were left to incubate for 30 minutes in a darkened room at a room temperature. The absorbance was measured at 517 nm with methanol as a blank sample. 0.5 mL of 0.5 mM DPPH dilution, diluted with 4 mL of methanol, was used as a control sample. The radical scavenging effect (%) or percent inhibition of DPPH radical was calculated according to the equation:

$$[(Ac - As) / Ac] \times 100$$

where As is the absorbance of the solution containing the sample at 517 nm, and Ac is the absorbance of the DPPH solution. The results were expressed as the IC_{50} value (mg/mL).

3. RESULTS AND DISCUSSION

In this study, total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity of methanolic extracts from fresh and dried nettle (*Urtica dioica* L.) were determined by Folin-Ciocalteu, DPPH, and FRAP methods. The results obtained are presented in Tables 1 and 2, where each sample of U-labeled extract is associated with an appropriate number indicating

the applied technique for its extraction (1-Soxhlet, 2-ultrasound-assisted and 3-maceration) and with designation (f), or (d), indicating whether the extraction was performed from a fresh or dry sample. Based on the results of measured phenolic compounds in extracts (Table 1), it can be seen that methanolic extracts obtained from dried nettle leaves had a high TPC (883-2323 mg GAE/100 g) depending on the extraction technique used, and TFC ranged from 1.42 to 3.05 mg QE/100 g. Comparing these results with the results of TPC (592 to 1963 mg GAE/100 g) and TFC (0.81-1.80 mg QE/100 g) of extracts obtained from fresh samples, it can be noticed that TPC and TFC values of extracts from dry samples were higher compared to the fresh samples, for all used extraction methods. The ranges of these values are comparable to the data from other study [40], where the results of TPC of methanolic extracts from dry leaves showed a high total phenolic content (15.5–21.8 mg/g). The higher values of TPC and TFC of extracts obtained from dried samples can be explained by the influence of drying of plant material on the preservation of phytochemicals in the final extract, because fresh plant samples are fragile and tend to deteriorate faster than dried samples [41]. In addition, drying can modify the physical micro structure of plant tissues, which leads to increased extraction yields [42]. The extraction efficiency will be enhanced by the small particle size due to the enhanced penetration of solvents and diffusion of solutes, and generally, the finer the particle size is, the better result the extraction achieves [43], which in the present research is accomplished by smaller particle size of milled dry plant material compared to chopped fresh material. From the aspect of efficiency of extraction methods, the highest contents of TPC and TFC were measured in the extracts obtained by Soxhlet extraction of dry and fresh nettle samples, and the lowest contents were measured in the extracts obtained by maceration. This is in line with outcomes of other researches, where within conventional methods Soxhlet had the highest extraction yield [44], that is, the higher content of total phenolic and flavonoids compared to those obtained by maceration and ultrasound-assisted extraction [45]. The higher efficiency of extraction of phenolic compounds by Soxhlet compared to maceration and ultrasonic extraction can be attributed to the positive effect of high temperature applied in the extraction process, since an increase in the extraction temperature can increase both solubility and mass transfer rate [28]. In addition, the large difference between the concentrations of analyte in the cell solution and in the solvent is maintained throughout, due to multiple digestion of a plant material and continuous extraction while a fresh portion of a solvent is delivered [46]. Despite the drawbacks of conventional methods, Soxhlet is considered as a reference method and generally is used for comparison with the more sophisticated methodologies recently developed [47].

Table 2 shows the results of antioxidant capacity of nettle methanolic extracts, measured by FRAP and DPPH assays. It is essential to perform more than one type of antioxidantcapacity measurement, because the antioxidant activity is a complex procedure usually happening through several mechanisms and is influenced by many factors, which cannot be fully described with one single method [48].

Table 1. Total phenolic and flavonoid content in nettle extracts

Extract	TPC [mg GAE/100 g]	TFC [mg QE/100 g]
U-1(f)	1963	1.80
U-2(f)	658	0.86
U-3(f)	592	0.81
U-1(d)	2323	3.05
U-2(d)	1293	1.78
U-3(d)	883	1.42

Table 2. Results of antioxidant capacity of extracts

Extract	FRAP value [µmol/100 g]	IC_{50} value [mg/mL]
U-1(f)	14436.97	0.35
U-2(f)	10011.71	0.61
U-3(f)	7899.03	0.84
U-1(d)	26523.07	0.25
U-2(d)	11358.94	0.55
U-3(d)	9277.31	0.70

Based on the results in Table 2, the lowest extract concentration required for inhibition of 50% DPPH radicals was achieved with extracts obtained by Soxhlet technique, ie. extracts both from fresh (0.35 mg/mL) and dried nettle samples (0.25 mg/mL) obtained by Soxhlet extraction method showed significantly higher antioxidant activity comparing with other extracts. High content of phenolic compounds may be responsible for their strong antioxidant activity [49]. The efficiency of the extraction methods, given the maximum antioxidant capacity of the

extracts, can be shown in the following order U-1> U-2> U-3 for dry and fresh samples, and the same order applies to the efficiency of obtaining extracts with the highest TPC and TFC, which is in favor of the previously stated.Linear regression analysis showed a direct correlation between total phenolic content and antioxidant activity of nettle extracts [50]. The results also showed that antioxidant capacity values were higher in the extracts from dry nettle compared to the extracts from fresh nettle leaf, for all extraction methods. This confirms that the drying and extraction methods are major factors contributing to radical scavenging activities of herbal plants and their extracts [51].

4. CONCLUSION

The results of different extraction techniques (maceration, ultrasound-assisted and Soxhlet extraction) of fresh and dried *Urtica dioica* leaves suggest that nettle can be used as a source of antioxidant phenolic compounds. Among the techniques used in the present research, Soxhlet provided methanolic extracts with the highest content of total phenolic compounds and flavonoids while their content in extracts obtained by maceration was the lowest. Total antioxidant capacity from both DPPH and FRAP assays showed similar trend. The results of the content of total phenolic compounds and flavonoids in the extracts and the antioxidant activity of the extracts indicate their significant correlation. Extracts obtained from dried nettle leaves showed significantly higher antioxidant activity compared to fresh leaves, in all extraction techniques used. The overall results of this study can provide important information, both in the selection of techniques for the extraction of phytochemicals for pharmaceuticals, and the method of optimal preparation of plant material for extraction. Future scope of the study, which would include more samples of *Urtica dioica* of different geographical origin, along with chemical analysis of plant material and extracts, would further contribute to defining the correlation of extraction of specific phenolic compounds with certain extraction methods.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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