EFFECT OF EXTRACTION TECHNIQUE ON THE CONTENT OF BIOACTIVE COMPONENTS AND ANTIOXIDANT ACTIVITY OF AQUEOUS EXTRACTS OF FRESH AND DRIED NETTLE (*URTICA DIOICA* L.)

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:
The samples of stinging nettle were collected during June in the Tuzla region. Aqueous extracts were prepared from fresh and dried leaves in order to determinate and compare content of bioactive components and antioxidant potential. Conventional soxhlet, ultrasound assisted extraction and traditional maceration extraction were used as extraction methods. Quantitative determination of phenols and flavonoids was carried out using spectrophotometric methods. Antioxidant activity of nettle aqueous extracts was determined using ferric reducing antioxidant power and DPPH free radical scavenging activity. Extracts obtained by Soxhlet extraction showed the highest total phenolic and flavonoid content and expected the highest antioxidant capacity, while extracts obtained by maceration gave the lowest results.

KEYWORDS: stinging nettle extract; bioactive components; extraction; antioxidant

INTRODUCTION

Stinging nettle (*Urtica dioica* L.) is a widespread perennial herbaceous plant. Urtica dioica and the closely related Urtica urens, as the most prominent members of the genus Urtica, are native to Europe, Africa, Asia and North America [1]. Eastern Germany, the former USSR, Bulgaria, the former Yugoslavia, Hungry and Albania are known as primary producers of stinging nettles [2]. Nettle, as one of the most undervalued of economic plants, has a long history of use as food, dye, cosmetic, and drug in folk veterinary and human medicine. It is widely recognized and used as an edible and highly nutritious vegetable, from young leaves that are added to soups or salads to dried leaves for winter use [3-5]. Beside its usage in human nutrition, nettle has been used all over the world for centuries in traditional medicine. This plant was used for the treatment of arthritis, anemia, rheumatism of the joints and muscles, hypertension, gastrointestinal diseasas, eczema and used as diuretics, astrigents, cleansing tonic, component of antidiabetic teas and blood purifier [6-10]. Studies showed the presence of different class of compounds in nettle including polyphenols, flavonoids, phenolic acids, alkaloids, carotenoids, choline, histamine, macro and microelements [7, 11-14]. Plants produce many chemical compounds, as a result of various biochemical pathways, that are biologically active. These components are found throughout the plant world and they can be shown to have an effect on human health. The presence of flavonoids and phenolic acids, as two major classes of phenolic compounds, enables plants to act as reducing agents, hydrogen donators and signlet oxygen quenchers. When plant posses high amounts of these compounds it has potent antioxidant activity leading to various defensive and disease fighting properties [15]. Lutein, β-carotene and their isomers were found to be the major carotenoids during all leaf maturity levels [5]. Nettle is also good source of vitamins C, K, and B groups vitamins and minerals, such as calcium, iron, magnesium, phoshorus, pottasium and sodium [2, 4]. Due to its content of bioactive components nettle has been subject of study for antioxidant, anti ulcer and anaglesic properties, which were confirmed to be present [16].

The aim of this study is to determine and compare a content of biological components and antioxidant

power of aqueous extracts derived from fresh and dried leaves of stinging nettle. There are available other studies that focus on aqueous extracts of Urtica dioica, from investigating biological compounds and antioxidant capacity to antihyperglycemic activity, diuretic and hypotensive effects [17-19].

It is important to mention that all these studies were done in different parts of the world, on different types of nettle extract and various experimental methods were applied on them. Factors such as type of extraction method, type of compounds and solvents, extraction efficiencies, the geographical and ecological status, the climate, seasonal and experimental conditions and other, all play a role and may explain the diversity of results in different studies [1]. This can be especially noticed in significant differences of the results in studies that showcase antimicrobial activity of nettle. Some studies revealed that nettle extracts had inhibitory effects on various Gram positive and Gram negative bacteria, while some noted that ethanol, methanol and organic solvent extracts were antimicrobial inactive [16, 20-24].

EXPERIMENTAL

All chemicals used were of analytical grade and were used as received without any further purification. Chemicals were purchased from Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, Missouri, USA).

SAMPLING AND PREPARATION OF MATERIALS FOR ANALYSIS

A nettle sample was harvested in the Tuzla area in June. The sample was cleaned and the leaves separated from the rest of the plant. The fresh leaves was immediately subjected to extraction. Part of the fresh leaves was dried for seven days at room temperature, in a dark and dry place. Fresh and dried nettle samples were separately ground in an electric mill before the extraction process.

PREPARATION OF EXTRACTS

Three methods were used for extraction: Soxhlet extraction, ultrasonic extraction and maceration. In all three cases, 10 grams of chopped 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. Ultrasonic 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, the extracts were filtered through filter paper. The obtained filtrates were stored in a dark and cool place before analyzing.

DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

Total phenolic were determined through the Follin-Ciocalteu test following the protocol [25] with some modifications. 200 μL of extracts was mixed with 2.54 mL of 10% Folin-Ciocalteu reagent. After 5 min 420 μL of 10% sodium carbonate was added. After incubation for 60 minutes at room temperature, the absorbance was measured at 765 nm, along with the prepared blank. Gallic acid was used as standard for the calibration curve ($y= 0{,}0042x + 0{,}0076$, $R^2 = 0{,}9998$). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrammes per 100 grams of nettle.

DETERMINATION OF TOTAL FLAVONOID CONTENTS (TFC)

Total flavonoid content in the extracts was measured by the previously described method [26], with some modification. 1mL of extract solution were mixed with 0.3 mL of 5% sodium nitrate. 0.3 mL of 10% aluminium chloride was added after 5 minutes. After 6 minutes incubation at room temperature, 1 mL of 1 M sodium hydroxide was added to the reaction mixture. Immediately the final volume was make up to 10 mL with distilled water. Absorbance of sample was determined 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 sample (mg/100 g) .

FERRIC-REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

The reducing power of the extracts, that reflected their antioxidant activity, was determined according to the protocol [27]. 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 ($y = 0{,}001x + 0{,}0698$; $R^2 = 0{,}9997$) and expressed in mol per gram of nettle sample.

DPPH RADICAL SCAVENGING ACTIVITY

2,2-diphenyl-1-picryl-hydrazyl (DPPH) method was performed according to earlier described method [28]. A series of solutions was made in tubes by adding different volumes of extract supplemented with up to 2 mL of methanol. 0.5 mL of 0.5 mM DPPH solution were 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 along with a methanol as 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:

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

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 are expressed as the IC_{50} value (mg/mL).

DETERMINATION OF BIOELEMENT CONTENT IN PLANT MATERIAL AND EXTRACTS

3 grams of dried nettle leaves material were topped with aqua regia and incubated for 16 hours. After 16 hours, the mixture was heated to reflux for 2 hours. The content of bioelements in such prepared parent sample was determined using ICP OPTIMA 2100 DV (Perkin Elmer) spectrometer. Aqueous extracts of plant material were directly analysed, withour prior preparation.

RESULTS AND DISCUSSION

Table 1 shows the results of the content of total phenolic and flavonoids (designated by U) in the aqueous extracts of fresh and dried nettle, obtained by three extracting techniques: conventional soxhlet, ultrasound assisted extraction and traditional maceration extraction (designated by numbers 1, 2 and 3). The notation (f) indicates the fresh sample, and (d) marks the dried sample. The results showed that extracts obtained by Soxhlet extraction have the hightest total phenolic and flavonoid content per gram of plant sample, for both – fresh and dry samples, while extracts obtained by maceration have the lowest content, leaving extracts derived from ultrasonic extraction in the middle of this rank. The Soxhlet extraction, as conventional technique, has been widely used for the extraction of bioactive compounds from various plant materials [29]. Within conventional methods applied for the Extraction of Bioactive Compounds from Lebanese Urtica dioica, it was found that soxhlet method had the highest extraction yield and the maceration method had the lowest one [30]. The higher total phenolic and total flavonoids content in the extracts obtained by Soxhlet extraction, compared to those obtained by maceration and ultrasonic extraction, is probably due to the solvent circulation flow until the complete depletion of plant material [31].

It is important to note that aqueous extracts of dried nettle samples showed higher values of total phenols and flavonoids compared to extracts obtained from fresh samples. This may be related to the fragility of fresh samples, which tend to deteriorate faster than dry samples [32]. Air drying at ambient temperature was used in many studies of nettle bioactive compounds for samples preparation [33-37] since does not force dried plant materials using high temperature; hence, heat-labile compounds are preserved [38].

Table 1. Content of total phenolic and flavonoids in nettle extracts

Extract	TPC [mgGAE/1g]	TFC [mgQE/1g]
U-1(f)	14,34	$9{,}52 \times 10^{-3}$
U-2(f)	11,67	$6{,}28 \times 10^{-3}$
U-3(f)	2,84	-
U-1(d)	63,59	$25{,}77 \times 10^{-3}$
U-2(d)	26,61	$14{,}98 \times 10^{-3}$
U-3(d)	12,23	$4{,}28 \times 10^{-3}$

Total phenolic and flavonoid contents of aqueous extract of stinging nettle have been studied earlier. The total phenolic content obtained through study published in 2014. were at 322.941±11.811 mgGAE/g extract value, while total flavonoids were 133.916±12.006 mg Catechin/g extract [15]. Reason why results are different from presented above is probably due to different method of extracting. Aim of this study was to compare results of three different extraction methods and of two types of samples, extracts of fresh and shade dried and to ease the process of choosing extraction technique.

ANTIOXIDANT ACTIVITY

Table 2. Results of antioxidant capacity and reduction ability of extracts obtained by DPPH and FRAP method

Extract	DPPH IC_{50} [mg/mL]	FRAP value [µmol/g]
U-1(f)	0,744	12171
U-2(f)	0,918	11257
U-3(f)	12,67	277,37
U-1(d)	0,122	61856
U-2(d)	0,145	46344
U-3(d)	0,292	9598,5

Antioxidant activity of aqueous extracts derived from nettle was determined using FRAP and DPPH methods, and results of antioxidant capacity are shown in Table 2. Instead of costly and inefficient separation

of each phenolic antioxidantan, total antioxidant power of a complex sample is often more meaningful to evaluate the health benefits, because of the cooperative action of antioxidants [39].

Extracts that were obtained by maceration had the lowest antioxidant capacity, while extracts obtained with Soxhlet extraction had the highest antioxidant capacity. The above data correspond to the content of total phenolics which are responsible for the antioxidant capacity of the samples. A significant correlation between the content of phenolic compounds in plants and the antioxidant capacity has been observed in many studies [40-44].

Once more, it was confirmed that extracts of dried nettle showed higher antioxidant capacity. To compare, Vitamin C with IC_{50} 0,03 mg/ml has much higher antioxidant capacity than obtained extracts by the present study. The nearest value to IC_{50} of Vitamin C is the value of DPPH noted in aqueous extract from shade dried nettle obtained by Soxhlet extraction. The antioxidant power of nettle extracts in present study can also be related to their phenolic content. This can be explained by the larger contact area between the dry sample and the extraction solvent. Due to the smaller and more homogeneous particle size of dried samples, more reducing compounds could be detected by extraction of dried leaves, than in the case of fresh plant material [33].

In earlier mentioned study [15], the IC_{50} of DPPH radical was 1.2 mg/mL. Even though values of total phenolics and flavonoids were higher, the above mentioned IC_{50} value is higher of every value in Table 2 of present study, except of DDPH result for extraction of fresh nettle obtained from maceration.

BIOELEMENTS CONTENT

Table 3 show the results of the content of bioelements in the extracts and in the parent sample which is listed in the table as nettle leaf. According to American Herbal Pharmocopoeia [45], the leaves of *Urtica dioica* are rich in mineral such as calcium, iron, magnesium, phosphorus, potassium and sodium, which is also confirmed by the results of analyzed elements in present study.

Table 3. Content of bioelements in extracts derived from netttle material and in parent sample (mg/kg)

Element	U-1(f)	U-2(f)	U-3(f)	U-1(d)	U-2(d)	U-3(d)	Nettle leaf
Fe	0,69	0,84	1,065	0,24	1,68	2,055	113,96
Cu	1,215	0,33	0,285	0,405	1,125	2,37	2,70
Co	0,045	0,045	0,06	0,06	0,06	0,06	0,10
Ca	3160,5	5037	4440	17115	12148,5	9009	17966,6
Na	144,6	91,2	125,4	283,05	114,45	174,7	381,00

In this study, the content of bioelements in extracts obtained from dry samples of nettle, in comparison with raw samples, generally had values closer to those in the parent sample. Figures 1 and 2 graphically show the extraction efficiency of bioelements from fresh and dried nettle leaves.

Among the three applied methods of extraction from dry nettle samples, soxhlet gave extracts with the contents of macroelements Ca and Na that are closest to those in the parenting sample. In other study [46], a higher content of macroelements Ca and Na was extracted from dried nettle leaves using maceration, than by Soxhlet and ultrasound-assisted techniques. Still, all three extraction methods in present study showed a significantly higher calcium content compared to sodium, in both fresh and dried samples, which is consistent with other reports of nettle, where calcium dominates compared to other macrominerals [3, 36, 47-48]. In addition to the highest content of macroelement calcium, the highest content among the analyzed microelements of the parent sample of nettle leaves had iron. In the comparison research of nutritional properties of Stinging nettle (Urtica dioica) powder with wheat and barley powders, calcium and iron levels in nettle powder were much higher than those from wheat and barley powders [49].

However, with regard to the extraction of Fe and Cu microelements in present study, maceration was more efficient than other applied methods. The results of the presence of cobalt in this study, both in the parent sample and in all obtained extracts from fresh and dried nettle leaves, showed the presence of cobalt in a lower content compared to other analyzed heavy metals, ie iron and copper. The order of Fe, Cu and Co content in nettle samples of this study is consistent with that in the study of concentrations of heavy metals in 54 samples of individuals and mixtures of medicinal herbal plants [50] where the order of decreasing mean total concentrations for these metals was Fe > Cu > Co, and cobalt was the least detected element.

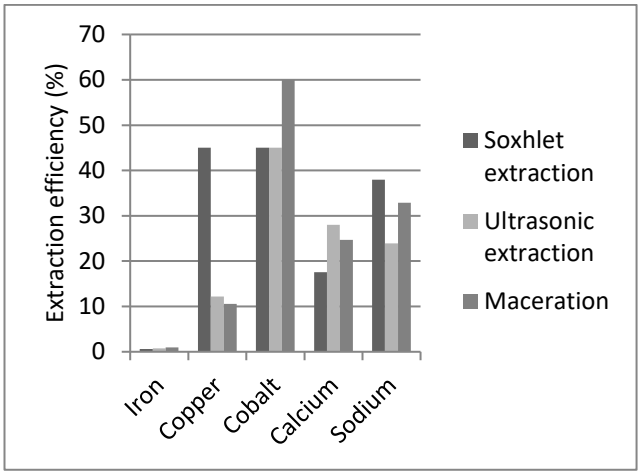

Figure 1. Efficiency of bioelements extraction from fresh netttle leaves by different extraction techniques

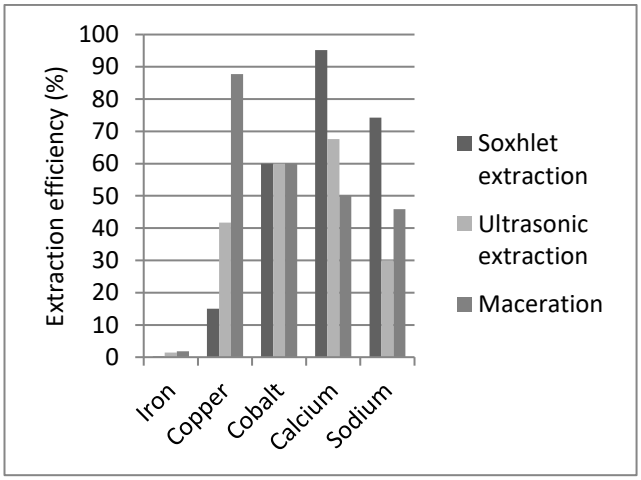

Figure 2. Efficiency of bioelements extraction from dried netttle leaves by different extraction techniques

CONCLUSION

Nettle leaf extracts showed different contents of bioactive compounds and bioelements, depending on the type of plant sample subjected to extraction, i.e. fresh or dry, as well as the extraction technique applied. In presented study, dry nettle leaf samples had yielded extracts with higher content of both bioactive compounds and bioelements, compared to fresh samples, regardless of the extraction method that has been used. Extracts obtained by Soxhlet extraction showed the highest total content of phenols and flavonoids, and accordingly the highest antioxidant capacity, while extracts obtained by maceration gave the poorest results. Conventional extraction techniques generaly have yielded extracts with a higher content of bioelements, where the Soxhlet method was more efficient in extracting macroelements Ca and Na from plant material, while maceration yielded extracts with a higher content of Fe and Cu microelements. The results of this research may contribute to the selection of the appropriate method of nettle samples preparation for extraction, as well as the appropriate extraction method, depending on the target bioactive compounds or bioelements in the plant material.

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