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THE INFLUENCE OF ULTRASOUND EXPOSURE TIME ON POLYPHENOL AND FLAVONOID YIELD AND ANTIOXIDANT POTENTIAL OF *SATUREJA MONTANA* L. EXTRACTS

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Abstract

Satureja montana L. (winter savory, Lamiaceae), as an evergreen perennial aromatic plant, contains essential oil, phenolic acids, flavonoids, labiatic acid, proteins, vitamins, and minerals. S. montana extracts have shown various pharmacological properties, such as antioxidant, antibacterial, antiviral, antitumor, anticatarrhal, and stimulant activities. Ultrasound-assisted extraction represents a modern extraction procedure that provides a higher extraction yield, fast kinetics, the use of a wide range of extraction solvents, a simple operation, and low costs in comparison to other novel techniques. In the present study, S. montana extracts were prepared using dried plant material (0.5 g), 50% ethanol as an extraction solvent (10 mL), and different extraction times (5, 15, 30, and 60 min) in an ultrasound bath. The extracts were analyzed via polyphenol and flavonoid yields, as well as DPPH radical scavenging activity. The total polyphenol content of the extracts rose up to the 15th min (from 12.7±0.7 to 20.6±0.4 mg gallic acid equivalents (GAE)/g of plant material) and after that reached the steady state (~21 mg GAE/g). The total flavonoid content was also rising with the increase in extraction time, from 4.34±0.28 mg catechin equivalents (CE)/g of plant material after 5 min to 8.19±0.36 and 8.42±0.30 mg CE/g after 15 and 30 min, respectively. However, the flavonoid concentration significantly decreased after 60 min of ultrasound-assisted extraction (7.38±0.32 mg CE/g). The extraction time significantly affected the DPPH radical scavenging capacity of the extracts: 65.4±1.3% after 5 min, 94.5±1.1% after 15 min, 87.0±2.4% after 30 min, and 91.6±2.0% after 60 min. According to the presented results, it can be concluded that 15 min represents the optimal ultrasound exposure time to obtain the extracts with the highest polyphenol and flavonoid yields, as well as DPPH antioxidant activity. The presented study shows the possibilities for the production of S. montana extracts with antioxidant compounds that can be potentially implemented in food, functional food, dietary supplements, pharmaceuticals, and cosmetics.

Key words: *Antioxidant activity, flavonoids, polyphenols, Satureja montana, ultrasound-assisted extraction.*

Introduction

Satureja montana L. (winter savory, *Lamiaceae*), as an evergreen perennial aromatic plant, contains essential oil (carvacrol, p-cymene, and thymol), phenolic acids, flavonoids, triterpenic acids, labiatic acid, proteins, vitamins, and minerals (Khan & Abourashed, 2011; Saeidnia et al., 2016). Winter savory is a bristly perennial subshrub with a woody base and oblong-linear leaves, native to the Mediterranean region and widely cultivated (Khan & Abourashed, 2011). The plant has been used for medicinal purposes due to its good antioxidant, stimulant, diuretic, mutagenic, anti-inflammatory, and many other beneficial biological effects. *S. montana* extracts have shown various pharmacological properties, such as antioxidant, antibacterial, antiviral, antitumor, anticatarrhal, and stimulant activities (Dewick, 2002; Khan & Abourashed, 2011). Food products in which winter savory oil and oleoresin are used include candy, baked goods, meat and meat products, and condiments and relishes (Khan & Abourashed, 2011). Ultrasound-assisted extraction provides enhanced extraction yield and quality, fast kinetics, a lower price, a simple device, as well as a wide range of used extraction solvents (Jovanović et al., 2017a). However, ultrasound waves can provoke the production of free radicals, so the extraction time for achieving the highest polyphenol (as antioxidants) yield should be optimized (Jovanović et al., 2017b). The identification and measurement of polyphenol components can provide important information on their role in the antioxidant capacity of the extracts, their influence on the quality of food, and their potential health benefits. By scavenging free radicals and lowering oxidative stress, antioxidants can postpone, limit, or completely halt the oxidation of substances, including proteins, lipids, and nucleic acids (Shukla et al., 2009; Zheng & Wang, 2001).

In the present study, *S. montana* extracts were prepared using dried herb and various extraction times using ultrasound-assisted extraction. The obtained extracts were characterized in terms of total polyphenol and flavonoid contents (TPC and TFC) and DPPH radical scavenging activity.

Materials and Methods

Plant material and reagents

Plant material used for polyphenol extraction was from a six year old cultivation trial with *S. montana* of a very rare chemotype of its essential oil, ct. thymol (~70%). Cultivation was conducted at the experimental field of the Institute for Medicinal Plants Research "Dr Josif Pančić", Serbia. The following reagents were used: Folin-Ciocalteu reagent, catechin, and gallic acid (Merck, Germany), sodium carbonate and ethanol (Fisher Scientific, UK), sodium nitrite and sodium hydroxide (Alkaloid Skopje, Macedonia), aluminum chloride (Kemika, Croatia), 2,2-diphenyl-1-picrylhydrazyl - DPPH (Sigma-Aldrich, USA), and ultrapure water.

Extraction

The extracts were obtained using 0.5 g of dried and grinded *S. montana* herb, 10 mL of 50% ethanol as an extraction medium, and various extraction times (5, 15, 30, and 60 min) in an ultrasound bath Sonopuls (Bandelin, Germany). The samples were filtered through filter paper after the extraction and the extracts were stored at 4°C until further analyses.

Determination of total polyphenol content of the extracts (Folin-Ciocalteu assay)

The total polyphenol content (TPC) of ethanol *S. montana* extracts was measured spectrophotometrically at 765 nm using the modified Folin-Ciocalteu method (Singleton et al., 1999). The results are expressed as milligrams of gallic acid equivalents per gram of plant

material (mg GAE/g).

Determination of total flavonoid content of the extracts

The total flavonoid content (TFC) of ethanol *S. montana* extracts was measured spectrophotometrically at 510 nm using a modified version of the Smolinski-Savi method (2017). The results are expressed as milligrams of catechin equivalent per g of plant material (mg CE/g).

Measurement of antioxidant capacity of the extracts (DPPH assay)

The antioxidant capacity of ethanol *S. montana* extracts was determined using the DPPH assay. The mentioned method was based on the procedure given by Mensor et al. (2001) with some modifications. The absorbance was measured at 517 nm and the results were expressed as a percentage of the neutralization of DPPH free radicals (%).
All spectrophotometric measurements were performed in a UV-1800 spectrophotometer (Shimadzu, Japan).

Statistical analysis

The statistical analysis was done by using analysis of variance (one-way ANOVA) and Duncan's *post hoc* test in STATISTICA 7.0. The differences were considered statistically significant at $p<0.05$.

Results and Discussion

The impact of different ultrasound-assisted extraction times (5, 15, 30, and 60 min) on TPC, TFC, and antioxidant activity of ethanol *S. montana* extracts were examined and the results are shown in Table 1.

Table 1. Total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant activity (DPPH assay) of ethanol Satureja montana extracts prepared using different ultrasound-assisted extraction times.

extraction time [min]	TPC [mg GAE/g]	TFC [mg CE/g]	DPPH neutralization [%]
5	12.7±0.7^{b*}	4.3±0.3^{c}	65.4±1.3^{c}
15	20.6±0.4^{a}	8.2±0.4^{a}	94.5±1.1^{a}
30	21.1±0.6^{a}	8.4±0.1^{a}	87.0±2.4^{b}
60	21.0±0.5^{a}	7.4±0.3^{b}	91.6±2.0^{ab}

**Values with different letters (a-c) in each row showed statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's post-hoc test; gallic acid equivalent, GAE; catechin equivalent, CE.*

According to the results of TPC of ethanol *S. montana* extracts, it can be concluded that extraction time significantly influenced polyphenol yield (Table 1). The polyphenol concentration of the extracts rose until the 15th min (from 12.7±0.7 to 20.6±0.4 mg GAE/g) and then reached a steady state (~21 mg GAE/g). The obtained results are in agreement with the literature data, where the concentration of the extracted biologically active components increased with prolonged exposure time. There are two levels in the release of polyphenols into the extraction medium: an initial increase in TPC values for 15 min and slow recovery after 60 min (Jovanović et al., 2017b; Nayak 2015). Nevertheless, prolonged exposure time can cause a

decrease in polyphenol yield because of enzymatic degradation and oxidation of polyphenol compounds and polymerization of insoluble components (Vergara-Salinas et al., 2012).

The extraction time had a statistically significant influence on the flavonoid concentration of ethanol *S. montana* extracts as well (Table 1). The TPC was rising with the increase in extraction time, from 4.34±0.28 mg CE/g after 5 min to 8.19±0.36 and 8.42±0.30 mg CE/g after 15 and 30 min, respectively. However, the flavonoid concentration significantly decreased after 60 min of ultrasound-assisted extraction (7.38±0.32 mg CE/g). It can be explained by the previously mentioned enzymatic degradation, oxidation, and polymerization as well as the thermosensitivity of flavonoids that occur when the prolonged exposure time is used (Jovanović et al., 2017b; Vergara-Salinas et al., 2012).

The extraction time also significantly affected the DPPH radical scavenging capacity of ethanol *S. montana* extracts: 65.4±1.3% after 5 min, 94.5±1.1% after 15 min, 87.0±2.4% after 30 min, and 91.6±2.0% after 60 min (Table 1). The results obtained in the DPPH assay followed the trend of TFC values, which is in agreement with the literature report where scavenging of free DPPH radicals was correlated with flavonoid concentration (Hirano et al., 2001).

According to the presented results, it can be concluded that 15 min represents the optimal ultrasound exposure time to obtain the extracts with the highest polyphenol and flavonoid yields, as well as DPPH antioxidant potential.

Conclusions

The aim of the present study was the preparation, chemical characterization, and examination of the antioxidant activity of ethanolic *S. montana* extracts using different extraction times in ultrasound-assisted extraction. The optimal extraction time to prepare the extracts with the highest polyphenol and flavonoid contents and DPPH radical scavenging potential was 15 min. Namely, prolonged exposure time caused a decrease in TFC and the antioxidant potential of *S. montana* extracts. The presented study shows the possibilities for the preparation of *S. montana* extracts with antioxidant compounds that can be potentially implemented in food, functional food, dietary supplements, and pharmaceutical and cosmetic formulations.

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Conflict of interest

The authors declare that they have no financial and commercial conflicts of interest.

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