Physicochemical properties of winter savory extracts prepared using ultrasound-assisted extraction

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This study aimed to assess the optimal extraction parameters, solvent-to-solid ratio, and extraction time, for ultrasound-assisted extraction of *Satureja montana* L. (winter savory) harvested from a 6-year-old experimental plantation. The optimization was performed in order to acquire extracts that are rich in the content of polyphenols (TPC), flavonoids (TFC), and proteins, and also exhibit strong antioxidant activity, while maintaining satisfactory physical characteristics. Besides this, the influence of UV irradiation on the physicochemical properties of the selected extracts was also analyzed. The TPC values increased with longer extraction time and higher ratio (ranging from 41.4 to 56.9 mg gallic acid equivalent (GAE)/g for the ratio 30:1, and 52.4–58.4 mg GAE/g for 40:1), and the TFC followed the same trend. Total protein content values increased with the increase in the solvent-to-solid ratio, while the extraction time had no significant influence. All used antioxidant assays showed better activities when a higher solvent-to-solid ratio has been performed. pH values ranged from 6.00 to 6.46. The extraction time and solvent-to-solid ratio did not significantly influence the conductivity, whereas the zeta potential was significantly affected. The highest density was detected in the extract acquired at a 40:1 ratio after 30 min (0.994 g/mL), and higher viscosity of the extracts attained at a 30:1 ratio compared to the parallels at a 40:1 ratio. According to the results of UV stability, UV light significantly affected the TPC, TFC, total proteins, ABTS radical scavenging, cupric, and ferric ion-reducing activities, with a positive effect on ferric ion-reducing potential, and the denisty. In conclusion, the extraction conditions that yielded the best phenolic-rich extract with good antioxidant potential and satisfactory physical properties were a 40:1 solvent-to-solid ratio and a 30 minute extraction time.

Key words: winter savory; ultrasound-assisted extraction; flavonoids; polyphenols; proteins

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ABBREVIATIONS

ABTS - 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
CE - catechin equivalent
CUPRAC -cupric ion reducing antioxidant capacity
DPPH - 2,2-diphenyl-1-picrylhydrazyl
FRAP - ferric reducing antioxidant power
GAE - gallic acid equivalent
TE - Trolox equivalent
TFC - total flavonoid content
TPC - total polyphenol content

1. INTRODUCTION

Satureja montana L. (winter or mountain savory) is a hardy evergreen perennial aromatic plant known for its culinary and medicinal properties. It is native to Southern Europe as far as Ukraine and Turkey, where it is represented in a diverse range of forms (Hammer and Junghanns, 2012). *S. montana* preferably grows in arid, sunny, stony, and rocky surroundings. Due to its high essential oil content, which may reach even 5 % in the case of fresh plant material (Jafari et al., 2016), it used to be commercially cultivated in the Balkans, Italy, and France. It is well-known that the chemical content and composition of the harvested plant material are determined by several factors including geographic location, altitude, climate, soil, and the ontogenetic stage of the plant, but also drying

and distillation/extraction methods. The chemical composition of *S. montana* extracts is characterized by the presence of polyphenolic compounds. Simple polyphenols, phenolic acids (derivatives of benzoic and cinnamic acids), lignans, lignins, coumarins, styrylpyrones, flavonoids, stilbenes, flavonolignans, and tannins are only a few of the many phytochemicals classified as plant phenolics (Dewick, 2001).

In climatic conditions of South Banat, Serbia, the essential oil content obtained by hydro-distillation of *S. montana* air-dried plant material was approximately 1.5 % (Radanović et al., 2018). In the oil of *S. montana*, carvacrol is commonly a major constituent, with 46.5–75 % of the total oil content (Lawrence, 1995), defining the oil as a carvacrol chemotype. Although rarely occurring, the oil could also be a thymol chemotype, with thymol content ranging from 48.3 %–69.4 % of the total oil content (Radanović et al., 2018). Whether the oil is of one or another chemotype, it is abundant in phenols, which provide the plant material with not only a strong, spicy flavor but also several beneficial properties. In addition to essential oil benefits, *S. montana* plant extracts also have several pharmacological benefits, including antibacterial, antiviral, antioxidant, antitumor, anticatarrhal, and stimulant effects (Jafari et al., 2016; Serrano et al., 2011).

Ultrasound-assisted extraction, microwave-assisted extraction, and supercritical fluid extraction belong to a group of novel extraction methods (Jovanović et al., 2017a). The ultrasound-assisted extraction (UAE) has previously been employed by Oalđe Pavlović et al. (2021), in order to obtain *S. montana* extracts for further determination of their biological activities, such as antioxidant capacity, antitumor potential and genoprotective activity in acellular system. Ultrasound waves are high-frequency sound waves that are above the human hearing threshold or 20 kHz. Since they are mechanical waves instead of electromagnetic ones, they can move through solid, gaseous, and liquid substances. By compression and rarefaction, these waves move through space. The expansions result in a decrease in the liquid's pressure. The creation of vapor bubbles happens when the pressure is higher than the liquid's tensile strength. Strong ultrasound fields cause cavitation, or the implosive collapse of these vapor bubbles (Jovanović et al., 2017a). As cavitation bubbles burst, the biomass's microporous particles are perturbed by macroturbulence, high-velocity interparticle collisions, and macroturbulence. A fast-moving stream of liquid is directed through the cavity at the surface by cavitation near liquid–solid contacts (Shirsath et al., 2012). These microjets' impingement causes surface peeling, erosion, and particle breakup, which makes it easier for bioactives to be released from the biological matrix. Through increased mass transfer via eddy and internal diffusion mechanisms, this impact improves extraction efficiency (Jovanović et al., 2017a). The main advantages of this extraction method include the possibility of large-scale extraction, easy use, higher extraction yield, reduced extraction period, and lower solvent consumption. On the other hand, the downsides of this method are: only the area around the ultrasonic emitter is where ultrasound is active, the attenuation of the ultrasonic wave is caused by the presence of a scattered phase, weak impact on oil extraction (Azmin et al., 2016).

Ethanol, as a polar solvent, is more hydrotropic and seeks to bond to the plant's water-soluble components causing the extraction or release of plenty of compounds (active and non-active) from the plant material. The resulting extract is hence less pure and often less potent, requiring more post-processing. The advantages of using ethanol as an extraction solvent are non-toxicity, more environmentally favorable medium, lower price, prolonged storage of the extract, extraction of huge amounts of plant material at once, and avoidance of dewaxing or winterization (Jovanović et al., 2017a). The disadvantages include its polarity, and thus the production of the extract with more water-soluble components, and its higher boiling point that requires a longer and slower recovery process (Zhao et al., 2022). The ethanolic extracts can be used in various ways, such as their independent action against different hazards and human pathogens, which can be viewed through their antioxidant, antibacterial, and antifungal effects, their incorporation into different types of carriers such as liposomes, freeze- or spray-dried particles, for the production of ingredients of various types of products in pharmaceutical, cosmetic, and food industries (Albu et al., 2004; Armendáriz-Barragán et al., 2016; Jovanović et al., 2021a).

Ultraviolet (UV) irradiation is used in all mentioned branches of the industry, for different reasons. In the food industry, it can be for pasteurization, the detoxification of aflatoxins, inactivation of infectious agents (Choudhary and Bandla, 2012). On the other hand, in the pharmaceutical industry, it is used for the determination of the mutation factor of this light on biofilms, the removal of certain chemicals from the wastewater, etc. (Ghafouri-Fard and Ghafouri-Fard, 2012). For these reasons, it is important to determine the effects that UV irradiation has on the extracts so that their use can be determined.

The aim of the present study was to optimize ultrasound assisted-extraction of polyphenols from winter savory by investigating factors of interest, extraction time and solvent-to-solid ratio. Chemical characterization of ethanolic extracts for savory included determination of total polyphenol and flavonoid contents (TPC and TFC, respectively), total proteins, antioxidant capacity (ABTS, DPPH, CUPRAC, and FRAP methods), whereas analyzed physical properties were extraction yield, pH value, conductivity, zeta potential, density, and viscosity. Since that UV irradiation is widespread in food, pharamceutical, and cosmetic industries, the UV stability of the extracts was examined as well.

2. MATERIALS AND METHODS

2.1. Plant material and reagents

Plant material used in this study originates from a six-year-old experimental plantation with *S. montana* (ct. thymol; thymol ∼70 %), established at the experimental fields of the Institute for Medicinal Plants Research "Dr. Josif Pančić", Pančevo, South Banat, Serbia. The plant material was harvested at full bloom, on September 14th 2021. It was air-dried at room temperature and stored until used for extractions.

For the extract preparation and the characterization, the following reagents were used: Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, gallic acid, catechin, methanol, Trolox, iron(II)sulfate, and iron(III)chloride were bought from Sigma Aldrich (USA), sodium-carbonate from Zdravlje (Serbia), sodium nitrite from Alkaloid Skopje (Macedonia), aluminum chloride, and trichloroacetic acid was from Kemika (Croatia), sodium hydroxide from NRK Inzenjering (Serbia), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-ABTS is from Roche Diagnostics GmbH (Germany), neocuproin is from Acros Organics (Belgium), monosodium phosphate, disodium phosphate, and phosphoric acid are from Merck (USA), cuprum chloride is from Fluka (Germany), ammonium acetate, and ethanol are from Zorka Pharma (Serbia), and they were all of analytical grade purity. The Coomassie Blue G250 was acquired from ThermoFisher Scientific (USA).

2.2. Preparation of the extracts

The ethanolic extracts from winter savory were prepared using 30 % ethanol as an extraction medium, two different solvent-to-

solid ratios (30:1 and 40:1 mL/g), and three different extraction times (5, 15, and 30 min) in an ultrasound bath (SONOREX SUPER RK 52 H, BANDELIN electronic, Germany, 230 V,~ 50/60 Hz, 0.9 A). Grinded dried herba and solvent are mixed and exposed to ultrasound waves. After the ultrasound-assisted extraction, the samples were filtered and stored at 4 °C until further analyses.

2.3. Determination of total polyphenol content

The spectrophotometric method, previously described by Zuhair et al. (2013) with a few modifications, was used to determine the total polyphenol content (TPC) in ethanolic *S. montana* extracts. Properly diluted sample (20 μL) and combined with 100 μL of the previously prepared Folin-Ciocalteu reagent (ratio of water:reagent was 2:1) in 1.5 mL of distilled water. This was followed by the addition of 2 mL of distilled water and 300 μL of a 20 % Na_2CO_3 solution. The absorbances were measured at a wavelength of 765 nm (1800 UV/Vis spectrophotometer, Shimadzu, Japan) after 120 min incubation period, in a dark environment, at room temperature. The Na_2CO_3 solution, distilled water, and Folin-Ciocalteu reagent were combined in the same ratio to create the blind sample, but the extracted sample was left out, instead, the solvent was used. Three parallel experiments were used to asses each sample. The calibration curve was created using a gallic acid solution. Results are given in terms of milligrams of gallic acid equivalent per gram of dried plant material (mg GAE/g).

2.4. Determination of total flavonoid content

The method previously described by Zuhair et al. (2013) was slightly modified for use in this research. In brief, 250 μL of the sample, which had been suitably diluted, was combined with 1250 μL of distilled water and 75 μL of a 5 % solution of $NaNO_2$. $AlCl_3$ solution (10 %, 150 μL) and 500 μL of a 1M NaOH solution were added after 5 min of incubation. After that, the mixture was diluted to a 3 mL volume. A 510 nm wavelength was used to measure the absorbance in comparison to the control sample, which consisted of distilled water, $NaNO_2$, $AlCl_3$, NaOH, and extraction solvent. The calibration curve was produced using catechin monohydrate. For each sample, TFC was assessed three times. Results are given as mg of catechin equivalent per gram of dried plant material (mg CE/g).

2.5. Determination of total proteins

The total proteins of the ethanolic savory extracts were determined using Bradford protein assay (Petrovic et al., 2019). The Bradford reagent (2.5 mL) was mixed with the extract (0.050 mL); the absorbance was read after 10 min at 595 nm. Albumin was used for the calibration curve and the results were expressed as mg of proteins per g of dried plant material (mg/g).

2.6. Determination of antioxidant capacity

2.6.1. ABTS assay

The ABTS assay was performed employing a slightly modified procedure that was previously described by Zuhair et al. (2013). The $ABTS^{\bullet+}$ solution was diluted to the absorbance value of 0.70±0.02 at a wavelength of 734 nm. An extract sample (20 μL, diluted with 50 % ethanol in a ratio of 1:9) was mixed with 2 mL of ABTS•+ solution and incubated for 6 min in the dark at room temperature. The assay determination was performed in three parallel tries. The scavenging capacity was calculated as:

$$\Delta A = A_c - A_x\,,$$

where Ac refers to the absorbance of 2 mL $ABTS^{\bullet+}$ solution and 20 μL of solvent; A_x is the absorbance of the sample. A calibration curve was calculated using Trolox (concentrations of 0.2-1 mM), and the antioxidant activity was expressed as μmol Trolox equivalents per g of plant material (μmol TE/g).

2.6.2. DPPH assay

The assessment of the $DPPH^{\bullet}$ scavenging activity was also performed according to a method reported by Zuhair et al. (2013), with some changes. The $DPPH^{\bullet}$ solution was prepared by dissolving 0.252 mg of DPPH powderin 9 mL of ethyl alcohol. After that, 2 mL of this solution was mixed with 20 μL of the extract (diluted with 50 % ethanol in a ratio of 1:9), and incubated for 20 min at room temperature, without the presence of light. The test was performed in parallel endeavors. The absorbance was measured at 517 nm, after which the scavenging activity (SC_{DPPH}) was determined according to the following equation:

$$SC_{DPPH} = \frac{A_c - A_s}{A_c} \times 100\,,$$

where A_c is the absorbance of 2 mL $DPPH^{\bullet+}$ solution and 20 μL of solvent; As is the absorbance of the sample, and the results are presented as IC_{50} (mg/mL), the concentration of the extract required to neutralize 50 % of $DPPH^{\bullet+}$ radicals.

2.6.3. Cupric Ion Reducing Antioxidant Capacity assay

In cupric ion reducing antioxidant capacity (CUPRAC) assay, the mixture was made by mixing 0.8 mL of the extract with 1 mL of $CuCl_2 \times 2H_2O$, 1 mL of neocuproine, and 1.2 mL of ammonium acetate buffer, pH~7 (Petrović et al., 2019). The sample was then incubated for 30 min at room temperature and in complete darkness before the absorbance at 450 nm was determined. For each extract, the assay result was verified three times. Using Trolox, the calibration curve for this approach was obtained. In terms of mol of Trolox equivalent (TE)/g of dried plant material, the obtained results are expressed.

2.6.4. Ferric Reducing Antioxidant Power assay

The ferric reducing antioxidant power (FRAP) assay is a usually performed antioxidant assessment method that measures the reduction of ferric ion (Fe^{3+})-ligand complex to the intensely blue-colored ferrous (Fe^{2+}) complex by antioxidants in an acidic medium (Zuhair et al., 2013). Phosphate buffer (1 mL) and the $K_3Fe(CN)_6$ solution (1 mL) are combined with 100 μL of the extract. The mixture is incubated for 240 minutes at 50 °C. Following the incubation period, 0.25 ml of 10 % trichloroacetic acid solution and 0.25 mL of the produced sample are combined. Next, 0.75 mL of water and 0.17 mL of $FeCl_3$ (0.1 % w/v) are added. All of the reagents and 30 % ethanol were used as a blank. Three parallel runs of the experiment were conducted, and the absorbance was measured at 750 nm. The results are given as μmol Fe^{2+}/g of dried plant material and were calculated using ferrous sulphate to create the calibration curve.

2.7. Determination of extraction yield

The weight of the dry extract following solvent evaporation and the weight of the dry plant material were compared to determine the extraction yield (EY) of *S. montana* ethanolic extracts, which was expressed as a percentage (%). The following equation was used to compute the extraction yield:

$$\text{Yield (\%)} = \frac{weight\ of\ dried\ sample}{weight\ of\ the\ initial\ sample} \times 100$$

2.8. pH value, zeta potential, and conductivity measurements

At a temperature of 25 °C, a pH analysis of the extracts was carried out using a pH Meter (HI 2210, Hanna Instruments, Italy).

The zeta potential and conductivity of the ethanolic savory extracts were examined by using photon correlation spectroscopy in Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., UK); each extract was examined in triplicate at 25 °C.

2.9. Density and viscosity measurements

The density of the ethanolic savory extracts was determined using Force Tensiometer K20 (Kruss, Germany). Each extract was examined in triplicate at 25 °C. Viscosity was measured using Rotavisc lo-vi device (IKA, Germany). Each extract was examined in triplicate at 25 °C.

2.10. UV stability study

In the UV stability study, a laminar flow cabinet (AC2-4G8, ESCo, Singapore) was used for the irradiation experiment. The selected extract samples (10 mL) in uncovered Petri dishes was exposed to UV-C irradiation (253.7 nm) for 30 min at 25 °C; subsequently, all measurements for physicochemical characterization were performed.

2.11. Statistical analysis

The analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test (STATISTICA 7.0) were used for the statistical analysis and the differences were considered significant at $p<0.05$, n=3.

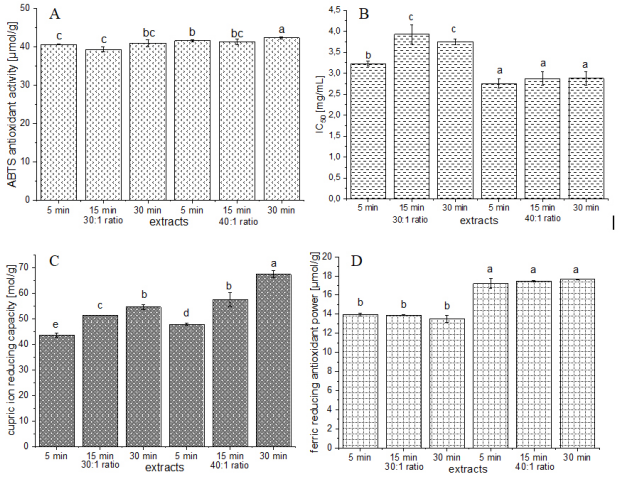

Fig. 1. Antioxidant capacity of *Satureja montana* L. (winter savory) extracts prepared using 30 % ethanol and ultrasound-assisted extraction at different extraction times and solvent-to-solid ratios: (A) ABTS, (B) DPPH, (C) CUPRAC, and (D) FRAP assays; values with different letters (a-e) in each column showed significant differences ($p<0.05$; n=3; analysis of variance, Duncan's *post-hoc* test).

3. RESULTS AND DISCUSSION

The ethanolic savory extracts were characterized via TPC, TFC, total proteins, antioxidant capacity, extraction yield, pH, zeta potential, conductivity, density, viscosity, as well as UV irradiation stability.

3.1. Total polyphenol, flavonoid, and protein contents of the extracts

With the aim to investigate the influence of the extraction time and solvent-to-solid ratio on the chemical composition of ethanolic *S. montana* L. extracts, the analyses of TPC, TFC, and total proteins were performed. The results are presented in Table 1.

The highest TPC was determined in the extract obtained at a 40:1 ratio after 30 min of ultrasound-assisted extraction (58.4±0.5 mg GAE/g), followed by the extracts prepared at a 30:1 ratio after 30 min and 40:1 ratio after 15 min (56.9±0.8 and 56.4±0.3 mg GAE/g, respectively). The extracts obtained after 5 and 15 min of the extraction at a 30:1 ratio have shown significantly lower polyphenol concentration (41.4±2.2 and 39.6±1.5 mg GAE/g). Additionally, the highest value of total flavonoids was achieved using a 40:1 ratio and 30 min of the extraction (25.6±0.7 mg CE/g), and again, the extract prepared using a 30:1 ratio and 5 min of ultrasound waves exposure was a poor source of flavonoids (12.4±0.1 mg CE/g), as in the case of TPC. Regarding the presented results, it can be concluded that both, extraction time and solvent-to-solid ratio have a significant influence on TPC and TFC. The literature shows that the increase in extraction time benefits the extraction of polyphenols and flavonoids, which might be caused by the length of time needed for these compounds to dissolve and diffuse through the plant cell membrane and into the solvent media via ultrasonic cavitation. However, prolonged exposure time in ultrasound-assisted extraction can cause the decomposition of recovered phenolic compounds (Zhou et al., 2013). Furthermore, in the literature, it can be seen that a high solid-to-solvent ratio is favorable for the extraction of phenolic compounds, which is in accordance with the mass transfer principle that is based on the concentration gradient between solid and solvent. A high solid-to-solvent ratio may encourage a growing concentration gradient, which would improve the diffusion rate and enable more solids to be extracted by the solvent. Additionally, as the amount of extraction solvent increases, the possibility of bio-active components coming into contact with the extracting solvent increases, resulting in increased leaching-out rates (Zhang et al., 2007). The TPC values of the ethanolic extracts of *S. montana* was 66.52 mg GAE/g in the research of Oalđe Pavlović et al. (2021), whereas, the detected TFC was 12.30 mg QE/g. Due to the fact that the plant material used in this study was cultivated, the differences between the results of Oalđe Pavlović et al. (2021) and those presented in this study may be attributed to differences in the chemical composition of *S. montana* collected from different localities.

On the other hand, the extraction time did not have a significant effect on the release of savory proteins into the extraction medium (Table 1). However, the solvent-to-solid ratio significantly influenced the total protein content in the extracts. Namely, all extracts prepared using a 40:1 ratio had higher protein concentration (from 20.0±0.5 to 20.5±0.5 mg/g) in comparison to the extracts prepared at a 30:1 ratio (from 17.3±0.5 to 18.3±0.5 mg/g). Our findings are in accordance with the literature, where it can be seen that ultrasound-assisted extraction can be used for obtaining a higher protein yield. The turbulence and shear force generated by ultrasound waves may be to blame for this impact, since they may efficiently disassemble the molecular structures of proteins, in order to create soluble proteins. However, it can be seen that the protein yield had decreased by the long extraction time (30 min) and high power of ultrasonic extraction, thus stating that the development of protein aggregates may be one of the reasons these results could have occurred (Lv et al., 2019). On the other hand, the increase in the protein yield, with a higher solid-to-solvent ratio is in accordance with the literature data (Tang et al., 2010).

Table 1. Total polyphenol content (TPC), total flavonoid content (TFC), total proteins, extraction yield, and pH values of *Satureja montana* L. (winter savory) extracts prepared using 30 % ethanol and ultrasound-assisted extraction at different extraction times and solvent-to-solid ratios.

Sample #	Time [min]	S-to-S ratio[a] [mL/g]	TPC [mg GAE/g]	TFC [mg CE/g]	Total proteins [mg/g]	EY [%]	pH
1	5	30:1	41.4±2.2 d	12.4±0.1 d	17.3±0.5 b	20.5±0.3 b	6.05±0.03 d
2	15	30:1	39.6±1.5 d	19.1±0.2 c	17.6±0.3 b	20.8±0.3 b	6.01±0.02 d
3	30	30:1	56.9±0.8 b	21.1±0.9 b	18.3±0.5 b	20.1±0.4 b	6.00±0.02 d
4	5	40:1	52.2±0.2 c	19.5±0.2 c	20.0±0.5 a	22.0±0.2 a	6.46±0.03 a
5	15	40:1	56.4±0.3 b	20.5±0.2 b	20.5±0.5 a	22.4±0.2 a	6.22±0.01 b
6	30	40:1	58.4±0.5 a	25.6±0.7 a	20.1±0.4 a	22.0±0.3 a	6.16±0.03 c

[a] Values with different letters (a-d) in each column showed significant differences (P<0.05; n=3; analysis of variance, Duncan's *post-hoc* test); S-to-S ratio, solvent-to-solid ratio; GAE, gallic acid equivalent; CE, catechin equivalent; EY, extraction yield.

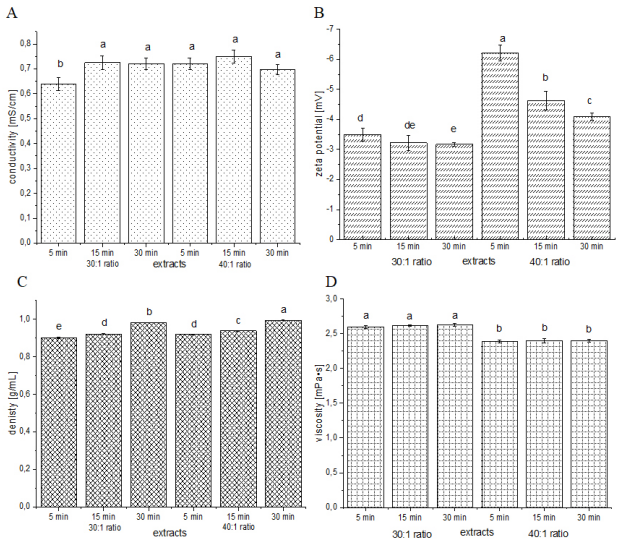

Fig. 2. (A) Conductivity, (B) zeta potential,(C) density, and (D) viscosity of *Satureja montana* L. (winter savory) extracts prepared using 30 % ethanol and ultrasound-assisted extraction at different extraction times and solvent-to-solid ratios; values with different letters (a-e) in each column showed significant differences (p<0.05; n=3; analysis of variance, Duncan's *post-hoc* test).

3.2. Antioxidant potential of the extracts

$DPPH^{\bullet+}$ radical scavenging potential of *S. montana* ethanolic extracts was expressed as IC_{50} value (Figure 1B). Namely, a lower IC_{50} value is related to higher antioxidant activity. Thus, the extracts obtained at a 40:1 ratio possessed the lowest IC_{50} values (from 2.75±0.11 to 2.88±0.17 mg/mL), followed by the extract prepared at a 30:1 ratio after 5 min (3.23±0.06 mg/mL). The extracts obtained at a 30:1 ratio after 15 and 30 min have shown the lowest activity for $DPPH^{\bullet+}$ radical neutralization (3.93±0.23 and 3.75±0.10 mg/mL, respectively). In the case of $DPPH^{\bullet+}$ radical scavenging activity, both factors (time and solvent-to-solid ratio) have shown significant impact, with the fact that prolonged extraction time caused the decrease in antioxidant capacity, while higher solvent-to-solid ratio led to higher DPPH neutralization by the extracts. The $DPPH^{\bullet+}$ radical neutralization ability of the extracts depends, in addition to polyphenols, also on the presence of saponins, alkaloids, terpenoids, and similar chemical compounds (Jadid et al., 2017). It can be seen in the literature that phenolic compounds undergo degradation when the period of ultrasound-assisted extraction is too long (Zhou et al., 2013). In the case of a higher content of polyphenols and flavonoids, there will be a greater ability to reduce $DPPH^{\bullet+}$ radicals.

As can be seen from Figure 1C, both, extraction time and solvent-to-solid ratio significantly influenced cupric ion reducing the antioxidant capacity of *S. montana* extracts, as in the case of $DPPH^{\bullet+}$ radical neutralization. Prolonged exposure to ultrasounds waves, as well as a higher amount of the extraction medium (at 40:1 ratio), have caused the increase in the release of the compounds responsible for cupric ion reducing potential. Thus, the highest antioxidant activity in the CUPRAC assay was measured in the extracts prepared at a 40:1 ratio after 30 min of ultrasound extraction (67.4±1.4 mol TE/g), which is correlated to the highest TPC and TFC in the mentioned extract (Table 1). According to the literature, the chemical compounds that show the highest CUPRAC assay are epicatechin gallate, epigallocatechin gallate, quercetin, fisetin, epigallocatechin, catechin, caffeic acid, epicatechin, gallic acid, rutin, and chlorogenic acid, in the exact order. The literature states that this is due to the position and quantity of hydroxyl groups and the degree of conjugation of the entire molecule, which allows easy electron transfer (Apak et al., 2007). The literature also shows that when a longer extraction period is employed, the CUPRAC assay values are higher (Uysal et al., 2019), which is in accordance with the findings of this paper. A higher solid-to-solvent ratio provides extracts with a higher CUPRAC value, which is in agreement with the findings in the literature (Mothapo, 2016). The work of Apak et al. (2007) states that there is a certain correlation between the TPC values obtained by the Folin-Ciocalteu method, which results in the occurrence of the highest CUPRAC value when the total polyphenols have the highest value.

According to the results from the FRAP assay (Figure 1D), it can be noticed that the extraction time did not have a significant effect on the ferric reducing capacity of winter savory extracts, whereas different solvent-to-solid ratios significantly influenced the mentioned variable. In specific, the extracts obtained at a higher ratio possessed significantly better potential ranging from 17.2±0.5 to 17.7±0.2 μmol Fe^{2+}/g, while the extracts prepared using a 30:1 ratio exerted reducing activity in a range from 13.5±0.5 to 14.0±0.2 μmol Fe^{2+}/g. The literature shows that the small changes in the FRAP assay with prolonged extraction time can be explained by the fact that some components that show antioxidant activity may be degraded due to the high temperature and effects of ultrasound (Zhou et al., 2013). On the other hand, a paper by Rawdkuen et al. (2016) states that the antioxidant activity significantly

Table 2. Physicochemical characterization of UV-irradiated winter savory (*Satureja montana* L.) extracts prepared using 30 % ethanol, 30:1 or 40:1 mL/g solvent-to-solid ratio during 30 min of ultrasound-assisted extraction.

Extract	S-to-S ratio	TPC[a]	TFC	Total proteins	ABTS	IC_{50} DPPH	CUPRAC	FRAP	Ext. yield	pH	Zeta potential	Conductivity	Density	Viscosity
	[mL/g]	[mg GAE/g]	[mg CE/g]	[mg/g]	[μmol TE/g]	[mg/mL]	[mol TE/g]	[μmol Fe^{2+}/g]	[%]		[mV]	[mS/cm]	[g/mL]	[mPa·s]
Control	30:1	56.9±0.8 a	21.1±0.9 a	18.3±0.5 a	40.9±0.9 a	3.75±0.07	54.5±1.0 a	13.5±0.3 b	20.1±0.4	6.00±0.02	-3.17±0.06	0.72±0.03	0.980±0.001 a	2.63±0.02
	40:1	58.4±0.5 a	25.6±0.7 a	20.1±0.4 a	42.3±0.3 a	2.88±0.17	67.4±1.4 a	17.5±0.1 b	22.0±0.3	6.16±0.03	-4.09±0.11	0.70±0.02	0.994±0.003 a	2.40±0.02
UV irrad.	30:1	28.9±2.6 b	15.1±1.0 b	13.4±0.1 b	15.2±0.3 b	3.83±0.29	50.2±1.3 b	16.1±0.6 a	20.2±0.5	5.99±0.02	-3.40±0.30	0.69±0.02	0.894±0.003 b	2.55±0.02
	40:1	30.1±1.6 b	16.7±1.2 b	16.5±0.5 b	23.2±1.3 b	2.65±0.12	63.1±0.6 b	19.5±0.6 a	22.4±0.4	6.13±0.03	-3.66±0.25	0.73±0.03	0.885±0.002 b	2.36±0.01

[a] The values with the different letters between the non-treated and UV-irradiated parallels mean that there is a significant difference between non-treated and UV-irradiated extracts; GAE, gallic acid equivalent; CE, catechin equivalent; TE, Trolox equivalent; IC_{50} (mg/mL), the concentration of the extract required to neutralize 50 % of DPPH radicals..

increased as the solvent concentration increased. This could be explained by the final equilibrium established between the solvent and solid concentrations in the plant matrix after a specific concentration level, which is yet to be reached. The sole limitation of the FRAP assay is that an aqueous testing apparatus is required, but it provides quick, repeatable findings. The reference antioxidant must therefore be a water-soluble substance like ascorbic acid, uric acid, or Trolox. The antioxidant capacity of ethanolic extracts from *S. montana* has previously been assessed by Serrano et al. (2011), and the IC_{50} value for the DPPH assay was 108.79 μg/mL, whereas the FRAP value was 93.60 μmol Fe^{2+}/g (Serrano et al., 2011).

The impact of the extraction time and solvent-to-solid ratio on the antioxidant capacity of ethanolic winter savory extracts was also examined using four antioxidant tests (ABTS, DPPH, CUPRAC, and FRAP assays) and the results are presented in Figure 1(A-D).

As can be seen from Figure 1A, $ABTS^{\bullet+}$+ radical scavenging capacity was the highest for the extract obtained at a 40:1 ratio and after 30 min (42.3±0.3 μmol TE/g) which is in agreement with the highest TPC and TFC determined in this extract (Table 1), followed by two other extracts prepared at the same ratio and different extraction times (41.6±0.3 and 41.2±0.7 μmol TE/g). Antioxidant capacity did not statistically differ in all extracts obtained at a 30:1 ratio and it was within a narrow range from 39.3±1.0 to 40.9±0.9 μmol TE/g. The effects of the solvent-to-solid ratio on antioxidant capacity have only been the subject of a Tan et al. (2011) study. However, it can be inferred that until it reaches an ideal level, antioxidant capacity increases as the solvent-to-solid ratio does. The trends of the TPC and TFC matched those seen in ABTS assays measuring radical scavenging ability. This result showed that the antioxidant capacity may be a result of both phenolic and flavonoid molecules (Tan et al., 2011). However, the extraction time did not influence the release of antioxidant compounds responsible for the neutralization of $ABTS^{\bullet+}$+ radicals. The ultrasonic-assisted extraction method that was employed in this work caused an increase in TPC and antioxidant activity up to achieving the equilibrium, while after that, prolonged extraction time did not result in the further release of polyphenol compounds. Namely, in our preliminary screening (data not shown), there was no statistically significant difference in TPC between the extracts obtained after 30 and 60 min. Phenolics, flavonoids, and anthocyanins may undergo chemical breakdown if subjected to a prolonged ultrasonic treatment at a high temperature (Ramić et al., 2015).

3.3. The extraction yield

The percentages of the extracted compounds from *S. montana* are presented in Table 1. The extraction yield depends on various factors, particularly on the parameters of the extraction procedures, such as extraction time, extraction medium, pH value, solvent-to-solid ratio, temperature, pressure, the presence of enzymes, etc. (Jovanović et al., 2017b). However, as can be seen from Table 1, the extraction time did not significantly affect the content of the extracted compounds from winter savory, while the solvent-to-solid ratio had a significant impact on the extraction yield. In specific, the extraction yield in the samples obtained using different extraction times varied in a rather narrow range (20.1±0.4-20.8±0.3 % for a 30:1 ratio and 22.0±0.2-22.4±0.2 % for a 40:1 ratio). Milenovic et al. (2002) have reported that the increase in solvent-to-solid ratio caused the increase in the extraction yield, which was also the case in *S. montana* extracts (Table 1). Although the extraction yield should be correlated to TPC, the percentage of the extracted substances from *S. montana* correlate with total proteins, but not with TPC. Namely, according to Jovanović

et al. (2021b), the content of lipids, sugars, and proteins, as ballast substances, causes the increase in the extraction yield as well.

3.4. pH value, conductivity, and zeta potential of the extracts

pH values, as an important parameter for future application or encapsulation of ethanolic *S. montana* extracts, are presented in Table 1. pH values of ethanol extracts varied in a range from 6.00±0.02 to 6.46±0.03 that are adequate values for the future drying and encapsulation processes, as well as application in pharmaceutical and cosmetic formulations.

The conductivity and zeta potential of ethanolic winter savory extracts are presented in Figures 1A and B.

Jurinjak Tušek et al. (2018) have reported that the conductivity of the extracts can be used as a predictor of their antioxidant capacity and the extracts that possessed a higher antioxidant potential showed a higher value of conductivity as well. The conductivity of *S. montana* extracts varied in a range of 0.70±0.03 and 0.75±0.03 mS/cm (Figure 2A), thus it can be concluded that no extraction time nor solvent-to-solid ratio possessed a significant influence on the conductivity. Therefore, in the case of ethanolic winter savory extracts, conductivity did not correlate with the antioxidant potential. Namely, since the conductivity is influenced by the presence of extraneous ions, analysis of the extract's antioxidant capacity using antioxidant assays is necessary.

Zeta potential (absolute value), as a measurement of the system stability, was significantly affected by the extraction time and solvent-to-solid ratio in the case of winter savory extracts. The zeta potential decreased with the prolonged extraction time and increased with a higher solvent-to-solid ratio (Figure 2B). The highest zeta potential of *S. montana* extracts was at a 40:1 ratio after 5 min (-6.21±0.27 mV), whereas the lowest was at a 30:1 ratio after 30 min (-3.17±0.06 mV). Determination of the extract's zeta potential is important from the aspect of its future application, including its potential encapsulation and use in water treatment. The zeta potential of plant extracts depends on extraction conditions, as well as extracted compounds, and absolute values vary from 2 mV to 15 mV (Skaf et al., 2021).

3.5. Density and viscosity of the extracts

The density and viscosity of ethanolic *S. montana* extracts are presented in Figure 2B. Density is the basic physical parameter frequently used in chemical and biochemical engineering, as the indispensable thermodynamic data for processes that including fluid flow, heat transfer, and mass transfer (Oroian et al., 2015). The density of the extracts varied in the range from 0.900±0.002 to 0.994±0.003 g/mL (Figure 2C). Density increased with the increase of extraction time and it was higher for the extracts obtained at a 40:1 ratio in comparison to their parallels obtained at a 30:1 ratio. The continuous increase in density with the prolonged extraction time is in agreement with the trend observed in polyphenol and flavonoid yields, where TPC and TFC rose as the extraction time increased (Table 1). Additionally, higher density measured in the extracts with a 40:1 ratio is correlated to the higher extraction yield, as well as a higher concentration of total polyphenols and flavonoids, compared to their 30:1 parallels (Table 1). Our results are in agreement with Mladenović et al. (2018) study where the correlation between density and extraction yield has been shown. Additionally, Florindo et al. (2014) reported that the density of the solution depends on its composition and surrounding temperature.

Viscosity is an important property of fluids that needs to be known for a wide variety of industrial and physicochemical processes, including catalysis, adsorption, distillation, extraction, etc. (Oroian et al., 2015). The viscosity values of ethanol winter savory extracts prepared using different times in ultrasound-assisted extraction were not different (2.60±0.02-2.63±0.02 mPa·s for a 30:1 ratio and 2.39±0.02-2.40±0.03 mPa·s for a 40:1 ratio, Figure 2D). Boros et al. (1993) have shown that extraction time had a significant influence on the increase of viscosity of *Secale cereale* extracts, but after a certain period of time, prolonged exposure to the extraction conditions caused the decrease of the extract viscosity. Probably, the exposure of *S. montana* plant material to the ultrasound waves should be extended to conclude if extraction time affects the viscosity of the extracts. Namely, endogenous hydrolytic enzymes are the most responsible for the decrease in extract viscosity, due to the hydrolysis of the viscous extracted compounds (Boros et al., 1993). Also, it should be indicated that the time between the preparation of the extract and the analysis of the extract's viscosity has a significant influence on the viscosity values. On the other hand, the solvent-to-solid ratio significantly affected the viscosity of the extracts; the extract prepared using the 1:30 ratio had significantly higher viscosity compared to the 1:40 ratio (Figure 2). According to the literature data, there is no correlation between viscosity and density, but both of the variables are affected by the temperature. Namely, the increase in temperature causes an increase in density and viscosity (Oroian et al., 2015). Apart from that, Singh and Singh (2011) have shown that viscosity is linearly correlated with surface tension, while surface tension is linearly correlated with density. However, in the case of winter savory extract, the extracts with higher density have shown lower values of viscosity (40:1 ratio). It can be explained by the occurrence of hydrolytic reactions in the higher amount of extraction medium containing 50 % of water, which causes the decrease in viscosity of the sample because of the hydrolysis of different extract components (Boros et al., 1993).

3.6. UV stability of the selected extracts

The selected *S. montana* extracts (samples with the highest TPC and TFC from both solvent-to-solid ratio levels) were exposed to UV irradiation for 30 min, and subsequently, physicochemical characterization of UV irradiated extracts was performed. The obtained results are presented in Table 2.

As can be seen from Table 2, UV irradiation has shown a significant impact on TPC, TFC, total proteins, ABTS radical scavenging, cupric and ferric ion reducing activities, with the fact that in ferric ion reducing potential it was a positive influence. Namely, the selected *S. montana* extracts possessed higher ferric ion-reducing activity in comparison to non-treated parallels. The explanation may lie in the UV-induced polymerization of polyphenols (Behboodi-Sadabad et al., 2017). According to the literature data, the mentioned reaction can form polymers of polyphenols with greater or lower antioxidant capacity. However, the new polymer compounds probably cannot be quantified in TPC and TFC analyses, but they can have an important role in the antioxidant potential of extracts (Jovanović et al., 2021a), in this case in ferric ion-reducing activity. On the other hand, the literature provides data stating that the content of some polyphenolic groups, which belong to flavonoids, such as orientin, apigenin-6,8-di-C-α-L-arabinopyranoside increases after exposure to UV light, whereas most phenolics content decreases with a prolonged exposure time, which can also explain the changes in antioxidant activity of the irradiated extracts (Del-Castillo-Alonso et al., 2020). The exception of increasing the TPC, TFC, and antioxidant assay values may be attributed to the fact that *S. montana* herb does not accumulate phenolics as a form of protection against UV damage (Edreva, 2005). The decrease in protein content may be attributed to the fact that UV irradia-

tion induces the production of reactive oxygen species (ROS), which cause subsequent oxidative damage (Roy, 2017).
On the other hand, UV irradiation did not influence the $DPPH^{\bullet+}$ radical scavenging capacity, extraction yield, and pH values of the selected extracts (Table 2). Previous work done by (Ali et al., 2012) investigated the effect of UV irradiation on the free radical scavenging activity, where they state that the increasing DPPH assay values may be attributed to the fact that the amount of secondary metabolites such as flavonoids and phenolic acids have become higher. On the other hand, Cvetkovic and Markovic (2008) found that UV irradiation leads to the degradation of carotenoids, resulting in lower DPPH values. Therefore, an assumption can be made, that the stable DPPH assay of the irradiated and non-irradiated extracts in this paper is a result of both of these effects, the degradation of carotenoids, with the increase in the flavonoid and phenolic acids content. Furthermore, UV irradiation has shown a significant effect only on one physical characteristic of *S. montana* extracts, i.e. density, while other examined variables (extraction yield, pH, zeta potential, conductivity, and viscosity) did not significantly change after UV irradiation (Table 2). The density values of UV-irradiated extracts were significantly lower in comparison to their non-treated parallels. It can be explained by the fact that numerous volatile active and non-active compounds have evaporated from the extract during the 30-min process of UV irradiation. Furthermore, water and particularly ethanol, as the parts of the extraction medium, has also evaporated (∼10 %), thus the evaporated amount of the solvent was compensated (but only pure ethanol) in order to avoid measurement errors in chemical analyses. Therefore, the loss of volatile components and the subsequent addition of pure ethanol could cause significant changes in extract density.

CONCLUSION

This paper aimed to determine the most favorable extraction conditions, solvent-to-solid ratio, and extraction time, for obtaining polyphenols-rich extracts with good antioxidant capacities and sufficient physical characteristics. Besides this, the influence of UV irradiation on the extracts acquired after an extraction time of 30 min, for both solvent-to-solid ratios (30:1 and 40:1) was examined. The results have shown an increase in the TPC, TFC, and total protein content values with a longer extraction period and a higher solvent-to-solid ratio. Following this, the ABTS and CUPRAC assays showed greater values for the extracts obtained after a longer extraction period and at higher solvent-to-solid ratios, which is in accordance with the polyphenol content, thus leading to the conclusion that these two types of antioxidant activities directly rely on the present amount of polyphenols and flavonoids. On the contrary, DPPH and FRAP assays were not influenced by the extraction period, while higher solvent-to-solid ratios led to a better antioxidant capacity. On the other hand, both the extraction yield and the pH value were not influenced by the extraction time, while the mentioned variables were higher for the extracts obtained at a higher solvent-to-solid ratio. The zeta potential decreased with the prolonged extraction time and increased with a higher solvent-to-solid ratio, while the mentioned parameters did not influence the conductivity of the extracts. The results of the physical characteristics presented in this study show that the solvent-to-solid ratio had a significant influence on density and viscosity, while only density rose with a prolonged extraction time. UV irradiation has shown a significant impact on TPC, TFC, total proteins, ABTS radical scavenging, cupric and ferric ion-reducing activities, as well as on density. For the development of food products or herbal remedies with antioxidant effects, *S. montana* raw material of standardized chemical quality, preferably originating from organic cultivation, and its extracts should be considered a suitable source. Future investigation should be aimed at the development of carriers for the bioactive compounds from winter savory extracts which provide the protection of sensitive compounds and controlled release of bioactives. The extract with the best biological activity was obtained using a 40:1 solvent-to-solid ratio and a 30 minute ultrasound-assisted extraction.

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