Optimization of the extraction process from *Satureja montana* L.: physicochemical characterization of the extracts

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Abstract

The presented study aimed to optimize polyphenol extraction from *Satureja montana* L. obtained from the experimental field of the Institute for Medicinal Plants Research "Dr Josif Pančić", Serbia, by varying the most important parameters for maceration, solid-to-solvent ratio, and extraction time. The obtained extracts were characterized regarding the total polyphenol content (TPC), total flavonoid content (TFC), antioxidant potential, extraction yield, conductivity, density, surface tension, and viscosity. The TPC and TFC were ~7 to 92 mg GAE (gallic acid equivalent)/g and 3.7 to 10.9 mg CE (catechin equivalent)/g, respectively. The highest extraction yield (86 %) as well as the highest antioxidant activities were obtained for the extracts prepared using a solid-to-solvent ratio of 1 g :50 cm^3. On the other hand, the extraction time did not have a significant influence. The highest conductivity was measured in the extract prepared at a 1 g :10 cm^3 ratio, while the highest density and surface tension were in the extract prepared at a 1 g :40 cm^3 ratio (941 kg m^{-3} and 29.0 mN m^{-1}, respectively). The highest viscosity was measured in the extract prepared at a 1 g : 20 cm^3 ratio (2.89 mPa·s). Our study shows the possibilities for the production of polyphenol-rich extract of *S. montana* which might be used in pharmaceutical, food, or cosmetic products.

Keywords: antioxidant activity; flavonoids; isolation; polyphenols; winter savory.

Available on-line at the Journal web address: http://www.ache.org.rs/HI/

ORIGINAL SCIENTIFIC PAPER

UDC: 615.451.1:544.344.016

Hem. Ind. **77(4)** 251-263 (2023)

1. INTRODUCTION

The *Satureja* genus comprises about 30 species [1], but due to the ease of cross-pollination, the same genus comprises also a great number of subspecies and varieties. However, only nine species from this genus may be found in the central and western Balkans, including Serbia [2]. One of the species is *Satureja montana* L., also known by its common name, winter savory. It is a perennial, semi-evergreen medicinal and aromatic species, which spontaneously grows in the sub-Mediterranean area [2], preferably in arid, sunny, stony, and rocky settings [3].

Ever since ancient times, *S. montana* has been used for medicinal purposes, as it exhibits good antioxidant, stimulant, mutagenic, anti-inflammatory, and many other beneficial biological activities [4]. In addition to its long-standing use in folk medicine, numerous scientific studies have already demonstrated its significant pharmacological efficacy, including antimicrobial activity against a variety of multidrug-resistant pathogens [5-9], as well as diuretic [10], antidiarrheal, and antispasmodic [11], anti-HIV-1 [12], antioxidant [6,13], anticholinesterase [8], and cytotoxic activities [14].

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 [15]. The identification and measurement of polyphenolic compounds can provide important information on the role of polyphenols as antioxidants, their influence on the quality of food, and potential health benefits.

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Paper received: 13 December 2022; Paper accepted: 21 August 2023; Paper published: 24 August 2023.
https://doi.org/10.2298/HEMIND221213020C

Due to their robust antioxidant properties and prevalence in a wide variety of regularly consumed fruits, vegetables, and spices, phenolic compounds have recently attracted significant attention as potential preventative measures against cancer and heart disorders [16]. It is widely acknowledged that the reactivity of the phenolic moiety causes phenolic compounds to serve as antioxidants. Biological activities of phenolic compounds have been the subject of several investigations [17-20] including anti-inflammatory, anti-ulcer, antispasmodic, antiviral, antidiarrheal, and antitumor effects [21].

By scavenging free radicals and lowering oxidative stress, antioxidants can postpone, limit, or completely halt oxidation of an oxidizable substrate. Antioxidants from outside sources are necessary in these circumstances to counteract the harmful effects of oxidative stress. A variety of putative pathways have been indicated as routes in which polyphenols might function as antioxidants. The most significant mechanism of their antioxidant activity is free radical scavenging, in which polyphenols can stop a free radical chain reaction, as well as suppress free radical formation by controlling the enzyme activity or chelating metal ions involved in free radical production.

Extraction is a method used in separation processes of target substances from a mixture. The mixture, specifically plant material, is brought into contact with a solvent, in which target substances are soluble. A widely known type of extraction of plant material is maceration. It entails immersing the plant material in a liquid (such as water, oil, alcohol, *etc*.) within an airtight container during different extraction times, depending on the plant material and liquid employed. The only disadvantage of this method in comparison to others is the extended period for obtaining the products [22,23]. As polyphenols from various plant materials have different structures, optimization of the extraction process is essential. Therefore, extraction of target compounds from particular plant material should be examined by varying extraction parameters, such as time, extraction medium, temperature, pH, solid-to-solvent ratio, *etc*. [23].

In the present study, the obtained extracts of *S. montana* were characterized *via* analyzing total polyphenol content (TPC), total flavonoid content (TFC), antioxidant potential (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and cupric ion reducing antioxidant capacity (CUPRAC) assays), conductivity, density, surface tension, and viscosity (Figure 1), with the aim to optimize the extraction time and solid-to-solvent ratio for achieving the highest extraction efficiency.

Figure 1. Flow diagram of the extraction process and the physico-chemical characterization of the extracts

2. EXPERIMENTAL

2. 1. Plant material and reagents

Plant material used in this study derives from a six year old plant cultivated during a trial with *S. montana* of a very rare chemotype of its essential oil, ct. thymol (≈70 wt.%). Cultivation was conducted at the experimental field of the Institute for Medicinal Plants Research "Dr Josif Pančić", Pančevo, Serbia. The following reagents were used: Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, gallic acid, catechin, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid-Trolox, iron(II)sulfate, and iron(III)chloride bought from Sigma Aldrich (USA), sodium-carbonate from Zdravlje (Serbia), sodium nitrite from Alkaloid Skopje (Macedonia), aluminum chloride, and trichloroacetic acid from Kemika (Croatia), sodium hydroxide from NRK Inženjering (Serbia), 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)-ABTS$^{\bullet+}$ from Roche Diagnostics GmbH (Germany), neocuproine from Acros Organics (Belgium), monosodium phosphate and disodium phosphate from Merck (USA), cuprum chloride from Fluka (Germany), ammonium acetate and ethanol from Zorka Pharma (Serbia).

2. 2. Preparation of the extracts

S. montana extracts were obtained in maceration (25 °C), using different extraction times (15, 30, 45, 60, 90, and 120 min) and solid-to-solvent ratios: 1 g :10 cm^3, 1 g :20 cm^3, 1 g :30 cm^3, 1 g :40 cm^3 and 1 g :50 cm^3 . Namely, grinded and sieved dried herb (the particle size of 0.5 mm) and extraction medium (50 % ethanol) are mixed and filtered after the extraction. The extracts were stored at 4 °C until further analyses.

2. 3. Determination of the total polyphenol content

The total polyphenol content (TPC) of the obtained *S. montana* extracts was determined by using a slightly modified Folin-Ciocalteu method, previously described in literature [24]. An appropriately diluted sample (0.02 cm^{-3}) was added to 1.5 cm^3 of distilled water and mixed with 0.1 cm^3 of the previously diluted Folin-Ciocalteu reagent (volume ratio of water : reagent is 2:1) [25]. Following this, 0.300 cm^3 of 20 wt.% Na_2CO_3 solution was added, and the mixture was filled up to the volume of 2 cm^3 using distilled water. After 2 h of the incubation in a dark place at room temperature, the absorbances were measured at a wavelength of 765 nm (1800 UV/Vis spectrophotometer, Shimadzu, Japan). The blank was obtained by mixing distilled water, extraction solvent (50 % ethanol), Folin-Ciocalteu reagent, and Na_2CO_3 solution, without adding the extract. Every sample was prepared in three parallels. Gallic acid solution was used to make the calibration curve. Results are expressed as mg of gallic acid equivalent (GAE) per g of the plant material.

2. 4. Determination of the total flavonoid content

The total flavonoid content (TFC) determination for the plant extracts was performed by using a modified version of the Smolinski-Savi method [26]. Appropriately diluted sample (0.25 cm^3) was mixed with 0.075 cm^3 of 5 wt.% solution of $NaNO_2$ and 1.250 cm^3 of distilled water. After the incubation time of 5 min, 0.15 cm^3 of 10 wt.% $AlCl_3$ solution was added, followed by the addition of 0.5 cm^3 of 1M NaOH solution [25]. The mixture was then diluted to a volume of 3 cm^3. The absorbance was measured relative to the blank sample at a wavelength of 510 nm. Catechin monohydrate was used to obtain the calibration curve. TFC was determined three times for every sample. Results are expressed as mg of catechin equivalent (CE) per g of the plant material.

2. 5. Determination of antioxidant capacity

2. 5. 1. ABTS assay

The analytical procedure published in literature [27] served as the foundation for the ABTS scavenging test with slight adjustments. The ABTS$^{\bullet+}$ solution was diluted in 96 vol.% ethanol to the absorbance value of 0.70±0.02 at a wavelength of 734 nm [19,20]. Extract sample (20 µl, diluted with 50 % ethanol in a volume ratio 1:9) was mixed with 2 cm^3 of ABTS$^{\bullet+}$ solution and incubated for 6 min in the dark at room temperature. The assay was performed in three

parallel tries. The scavenging capacity was calculated as $\Delta A = A_c - A_x$, where A_c refers to the absorbance of 2 cm^3 ABTS$^{\bullet+}$ solution and 20 µL of the solvent; A_x is the absorbance of the sample. A calibration curve was calculated by using Trolox (concentrations of 0.2-1 mM, $y = 0.374x$, where y was ΔA and x was concentration), and the antioxidant activity was expressed as µmol Trolox equivalents (TE) per g of the plant material.

2. 5. 2. DPPH assay

The DPPH assay was performed according to the procedure previously published [28] with some modifications. The DPPH$^{\bullet}$ solution was prepared by dissolving 0.252 mg of DPPH$^{\bullet}$ in 9 cm^3 of ethanol. After that, 2 cm^3 of this solution was mixed with 20 µl of the extract (diluted with 50 % ethanol in a ratio 1:9), and incubated for 20 min at room temperature, without the presence of light [25]. The absorbance was measured at 517 nm, after which the scavenging activity ($SC_{DPPH}{}^{\bullet}$) was determined according to $SC_{DPPH}=/(A_c-A_s) / A_c) 100$, where A_c is the absorbance of 2 cm^3 DPPH$^{\bullet}$ solution and 20 µL of solvent; A_s is the absorbance of the sample, and the results are presented as IC_{50} (mg cm^{-3}), *i.e*. the concentration of the extract required to neutralize 50 % of DPPH$^{\bullet}$ radicals. Values of IC_{50} were calculated from the calibration curve prepared from five different concentrations of *S. montana* extract (0.2-2 mg cm^{-3}) and inhibition of DPPH radicals, %. The analyses were performed in triplicates.

2. 5. 3. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay is based on the capacity of the sample to reduce Fe^{2+} contained in the potassium-hexacyanoferrate complex to Fe^{3+}. Namely, 10 mL of the extract is mixed with 1 cm^{3}of phosphate buffer and 1 cm^3 of the $K_3Fe(CN)_6$ solution [29]. The mixture is incubated at 50 °C for 4 h. After the incubation, 0.5 cm^3 of the prepared sample is mixed with 0.25 cm^{-3} of 10 vol.% trichloroacetic acid solution, and following that, 0.75 cm^3 of H_2O and 0.17 cm^3 of $FeCl_3$ (0.1 % m/v) were added. The negative control contained all reagents, except the extract. The assay was determined in three parallel tries, while the absorbance was measured at 750 nm. The calibration curve was obtained using ferrous sulphate, and the results are expressed as µmol Fe^{2+}/g of the plant material.

2. 5. 4. Cupric ion reducing antioxidant capacity assay

For the cupric ion reducing antioxidant capacity (CUPRAC) assay, a mixture was prepared by adding 1 cm^3 of $CuCl_2{\times}2H_2O$, 1 cm^3 of neocuproine, and 1.2 cm^3 of ammonium acetate buffer (pH ~7) to 0.8 cm^3 of extract [30]. The sample was then incubated at room temperature, in the dark for 30 min, after which the absorbance was measured at 450 nm. The assay determination was repeated three times for every extract. The calibration curve for this method was obtained using Trolox. The acquired results are expressed as mol of Trolox equivalent (TE) per g of the plant material.

2. 6. Determination of the extraction yield

The extraction yield (*EY*) was calculated as *EY* / % =((weight of the dried sample)/(weight of the initial sample)) 100 [31].

2. 7. Conductivity measurement

Conductivity of the extracts was determined by photon correlation spectroscopy (PCS) using the instrument Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., UK). Each sample was measured three times at room temperature.

2. 8. Density, surface tension, and viscosity measurements

Density and surface tension of *S. montana* extracts were measured by using silicon crystal (as the immersion body) and Wilhelmy plate, respectively, in the Force Tensiometer K20 (Kruss, Germany). Each sample was examined three times at room temperature.

Viscosity was determined by using the Rotavisc *lo-vi* device equipment with a VOL-C-RTD chamber, VOLS-1 adapter, and spindle (IKA, Germany). Each sample was examined three times at room temperature.

2. 9. Statistical analysis

In the present study, the statistical analysis was performed by the analysis of variance (one-way ANOVA) followed by the Duncan's *post hoc* test using the statistical software, STATISTICA 7.0 (TIBCO Software Inc., CA, USA) [23]. The differences were considered statistically significant at p <0.05, n = 3.

3. RESULTS AND DISCUSSION

Different *S. montana* extracts, obtained by using 50 vol.% ethanol at different extraction times and solid-to-solvent ratios in maceration, were examined in terms of chemical (TPC, TFC, and antioxidant potential) and physical characteristics (extraction yield, conductivity, density, surface tension, and viscosity).

3. 1. Total polyphenol and flavonoid contents in the extracts

Polyphenols, and particularly flavonoids, are the chemical components that most significantly contribute to antioxidant capabilities of extracts obtained from plant materials. As can be seen in Table 1, the extraction time had a statically significant influence on polyphenol contents in *S. montana* ethanol extracts, but not at all levels. Namely, in the first 45 min of the maceration, TPC did not differ significantly (~32 mg GAE/g), whereas the polyphenol concentration significantly rose at 60 min (34.0±0.3 mg GAE/g) and reached a plateau (results were not statistically different after 90 and 120 min, 34.6±0.7 and 34.4±0.8 mg GAE/g, respectively). The obtained results are comparable to the previous findings [25] where the highest TPC in *Thymus serpyllum* extracts was achieved at 60 min in maceration. According to the literature, concentrations of the extracted bioactive compounds are increased with the prolonged extraction time. Namely, in polyphenol extraction, there are two levels: (1) an initial increment of TPC during 15 min and (2) slow extraction after 60 min [23,32]. However, further lengthening of the extraction time can reduce the TPC, due to enzymatic degradation and oxidation of polyphenols in the aqueous phase (the extraction medium in the case of *S. montana* extracts contains 50 vol.% of water), as well as polymerization of insoluble molecules [33].

The extraction time had a statistically significant impact on total flavonoids as well (Table 1). The highest flavonoid yield was achieved after 30 min of maceration (8.0±0.1 mg CE/g) and it stayed constant up to 90 min (8.1±0.9 mg CE/g).

Table 1. *Total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant capacity (ABTS, DPPH, FRAP and CUPRAC assays) of Satureja montana L. extracts obtained using different extraction times and solid-to-solvent ratios; 50 vol.% ethanol and maceration*

Extraction time, min (solid-to-solvent ratio of 1:20)	TPC, mg GAE/g	TFC, mg CE/g	ABTS, μmol TE/g	DPPH IC_{50}, mg cm^{-3}	FRAP, μmol Fe^{2+}/g	CUPRAC, mol TE/g
15	32.6±0.8^{b*}	6.7±0.2^{b}	229.5±29.2^{c}	4.01±0.24^{b}	14.2±0.5^{ab}	61.1±1.9^{a}
30	31.7±1.8^{b}	8.0±0.2^{a}	294.1±5.1^{a}	3.71±0.26^{ab}	14.0±0.4^{ab}	56.3±2.1^{b}
45	32.4±0.5^{b}	7.9±1.0^{a}	287.7±5.5^{a}	3.26±0.21^{a}	14.3±0.2^{a}	51.5±1.5^{c}
60	34.0±0.3^{a}	8.1±0.9^{a}	285.7±8.4^{a}	3.32±0.23^{a}	14.3±0.0^{a}	43.3±1.2^{d}
90	34.6±0.7^{a}	7.6±0.3^{a}	290.4±6.2^{a}	3.53±0.21^{a}	13.5±0.5^{b}	33.9±2.2^{e}
120	34.4±0.8^{a}	5.4±0.4^{c}	270.1±4.1^{b}	3.47±0.26^{a}	13.8±0.1^{b}	33.7±2.5^{f}
Solid-to-solvent ratio, g cm^{-3} ** (extraction time 30 min)						
1 : 10	7.1±0.2^{d}	3.7±0.1^{d}	147.3±1.4^{d}	6.63±0.02^{d}	7.0±0.4^{e}	15.0±0.3^{e}
1 : 20	34.0±0.3^{c}	8.1±0.9^{b}	285.5±8.1^{c}	3.32±0.03^{c}	14.3±0.0^{d}	43.3±1.2^{c}
1 : 30	50.1±1.1^{b}	10.4±0.3^{a}	426.7±5.4^{a}	2.71±0.07^{a}	20.3±0.3^{c}	37.3±1.4^{d}
1 : 40	63.6±2.4^{a}	10.9±0.3^{a}	378.2±5.1^{b}	3.04±0.15^{b}	26.7±0.2^{b}	50.4±1.1^{b}
1 : 50	52.2±2.5^{b}	5.9±0.1^{c}	441.2±17.3^{a}	3.06±0.13^{b}	34.2±0.1^{a}	78.9±1.2^{a}

*Values with different letters (a-f) in variable group showed statistically significant differences (p <0.05; n = 3; analysis of variance, Duncan's *post-hoc* test); 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; **the solid-to-solvent ratio represents the mass of plant material per volume of the extraction solvent

However, a significant drop in the TFC can be noticed after 120 min (5.4±0.4 mg CE/g), probably due to the previously mentioned degradation, oxidation, and polymerization reactions that occur during extended extraction time [33]. These findings are in accordance with the literature, where the extraction times were varied in a range from 30 to 105 min,

resulting in the maximum values for both TPC and TFC obtained after a 60 min maceration process. Further extension of the extraction time over 60 min was shown to induce a decrease in both TPC and TFC values [34-36].

As was expected, the solid-to-solvent ratio had a statistically significant influence on the TP yield in all examined levels (Table 1). The lowest TPC was measured in the extract obtained using a 1 g : 10 cm^3 ratio (7.1±0.2 mg GAE/g), because of the high plant material content and thus higher viscosity of the extraction surrounding. Therefore, diffusion of polyphenolic compounds through the extraction solvent was very slow, which resulted in a lower TP yield. Further, polyphenol concentration was increased continuously with the decrease in the solid-to-solvent ratio and reached the maximum at the 1 g : 40 cm^3 ratio (63.6±2.4 mg GAE/g). This phenomenon can be explained by high solubility of polyphenolic compounds in the ethanol/water mixture, particularly glycoside forms (glucoside, fructoside, glucuronide, *etc*.). Namely, a higher quantity of the extraction medium provides faster polyphenol diffusion and recovery [23,37]. However, in the case of a 1 g : 50 cm^3 ratio, the sample added to the reaction mixture in the Folin-Ciocalteu test is quite diluted, and thus showed significantly lower TPC (52.2±2.5 mg GAE/g), which implies that the Folin-Ciocalteu reagent is not sensitive enough for the testing of samples with lower concentrations of polyphenols. Additionally, the TF yield was also increasing continuously with the decrease in the solid-to-solvent ratio up to 1 g : 30 cm^3 and 1 g : 40 cm^3 ratios (10.4±0.3 and 10.9±0.3 mg GAE/g, respectively), while there was a significant drop in the flavonoid amount at the 1 g : 50 cm^3 ratio (5.9±0.1 mg GAE/g), as in the case of polyphenol content. Despite the fact that the extracted amount of the target compounds, calculated per g of the plant material, should reach a plateau approaching the value reached in the infinite amount of the liquid solvent, in the Folin-Ciocalteu method, 20 µl of the liquid sample (the same volume for every employed solid-to-solvent ratio) was added to the reaction mixture. The added extract sample was already diluted due to the higher amount of the extraction medium, therefore, the determined flavonoid concentration was lower. Namely, in the case of *S. montana* extracts, a higher quantity of the extraction medium did not provide higher polyphenol and flavonoid concentrations in the liquid extract. Since the aim was the preparation of the liquid extract with the highest polyphenol and flavonoid contents using the lowest amount of plant material, the comparison of different solid-to-solvent ratios was carried out after the calculation of the amount of polyphenol and flavonoid contents per g of the plant material.

The gradual increase in the TP yield with the decrease in the solid-to-solvent ratio from 1 g : 10 cm^3 to 1 g : 40 cm^3 is also in accordance with the literature, where it is stated that the highest polyphenol yield values were acquired at a ratio of 1 g : 30 cm^3 [18,23]. This is also in agreement with the findings of G. D'Alessandro *et al.* [38], in regards to the increase in TPC and TFC values, with the change in the solid-to-solvent ratio, which was in this paper up to the 1 g : 40 cm^3 ratio.

3. 2. Antioxidant capacity of the extracts

The antioxidant potential of various *S. montana* extracts was determined by using four antioxidant assays (ABTS, DPPH, FRAP, and CUPRAC tests) and the results are presented in Table 1.

$ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging activities significantly rose after 15 min of maceration and were constant during 90 min, but at 120 min maceration period a significant drop only in the ABTS antioxidant activity can be noticed, which is in correlation to the lower measured TF yield (Table 1). The yield of polyphenols and flavonoids, as the main plant antioxidants in the solvent, greatly increased with the extension of the extraction duration from 15 to 90 min, as demonstrated in the study. Nevertheless, despite the longer extraction time of up to 120 min, the yield of TP stayed constant or even decreased. According to the literature, a final equilibrium between the solute concentrations (in this case antioxidants) in the solid matrix and the solvent may be attained after a specific time, could explain these phenomena well [39]. On the other hand, the extraction time did not have a statistically significant effect on the ferric reducing antioxidant power of *S. montana* extracts (Table 1). This result can be explained by the fact that apart from polyphenols, other plant compounds present in the extracts, such as unquantified phenolics and/or synergism that exists between these compounds, and other types of compounds that are characterized by a much larger molecular weight, exhibit ferric reducing antioxidant potential [40]. Namely, the mentioned substances have different solubility in various ethanol-water mixtures, thus their release can take place very quickly in the extraction medium, already at the initial phase of the extraction. Therefore, prolonged extraction time did not influence the ferric reducing antioxidant

potential. However, in the case of cupric ion reducing antioxidant capacity, extended extraction time had a significantly negative impact. In specific, antioxidant capacity was the highest after 15 min and continuously decreased up to 90 min of maceration. Some flavonoids do not contain the necessary chelating functional groups for aluminum binding used in the determination of flavonoid contents. On the other hand, the mentioned flavonoids can be responsible for the cupric ion-reducing antioxidant potential of the extracts. Therefore, this can explain a high value of the antioxidant capacity of the extract in the CUPRAC assay and a lower value of the flavonoid content and *vice versa* [41]. Park *et al.* [42] reported that the limited correlation between the CUPRAC assay results and the flavonoid content was attributable to the nature of the measuring technique; a high flavonoid content as measured by aluminum chloride colorimetry does not necessarily represent a strong antioxidant capacity. When measuring flavonoids, the $AlCl_3$ colorimetric assay [43] does not measure flavonoids that do not contain the necessary chelating functional groups for Al^{3+} binding. As a general rule, flavones and flavonols react with Al^{3+}, but flavanones and flavanonols do not complex to the same degree (*e.g.* chrysin, apigenin, luteolin, *etc*.) [43]. Additionally, some flavonoids and phenolic acids are quite sensitive to oxygen, light, or organic solvent, and susceptible to degradation under prolonged exposure times [44].

Further, the extracts prepared using 1 g : 30 cm^3 and 1 g : 50 cm^3 ratios exhibited the highest ABTS radical scavenging activity, followed by the extract obtained at the 1 g : 40 cm^3 ratio, while the extract prepared by using the 1 g : 30 cm^3 ratio has shown the highest DPPH neutralization potential (*i.e*. the lowest IC_{50}), followed by the extracts obtained at 1 g : 40 cm^3 and 1 g : 50 cm^3 ratios (Table 1). The highest ferric and cupric ion reducing antioxidant potential was measured in the extract obtained using the 1 g : 50 cm^3 ratio. The lowest antioxidant activity in all four assays was detected in the extract obtained at the 1 g : 10 cm^3 ratio, which is in correlation to the lowest polyphenol and flavonoid extracted amounts. The effects of the solid-to-solvent ratio on antioxidant capacity have only been the subject in several research studies [18,19]. But, nevertheless, it can be concluded that there is an increase in the antioxidant activity due to the decrease in the solid-to-solvent ratio until the optimal level is reached. Keeping in mind that polyphenols and flavonoids are the chemical compounds responsible for the antioxidant capacity of the extracts, and both TFC and TPC values being higher with the lower of the used solid-to-solvent ratio, it is logical for the 1 g : 10 cm^3 ratio to show the least favourable antioxidant characteristics [45].

3. 3. The extraction yield

The efficiency of the procedure used to extract specific active components from the plant material employed, such as polyphenols, tannins, flavonoids, proteins, and so forth, is indicated by the extraction yield. The extraction time did not have a statistically significant effect on the extraction yield at the solid-to-liquid ratio of 1 g : 20 cm^3 , which varied in a range from 53.6±2.8 to 57.3±3.0 % for the extracts obtained at different extraction times (Table 2).

Table 2. *Extraction yield (EY), conductivity (G), density (ρ), surface tension (γ), and viscosity (η) of Satureja montana L. extracts obtained using different extraction times and solid-to-solvent ratios; 50 % ethanol and maceration*

Extraction time, min (solid-to-solvent ratio of 1:20)	*EY* / %	*G* / mS cm^{-1}	*ρ* / kg m^{-3}	*γ* / mN m^{-1}	*η* / mPa·s
15	56.4±2.5^{a}	0.59±0.03^{ab*}	875±3^{b}	27.8±0.2^{a}	2.85±0.02^{a}
30	53.6±2.8^{a}	0.61±0.01^{a}	878±2^{b}	27.6±0.8^{a}	2.87±0.01^{a}
45	57.3±3.0^{a}	0.57±0.01^{b}	877±2^{b}	27.4±0.3^{a}	2.90±0.04^{a}
60	56.8±1.9^{a}	0.55±0.01^{b}	898±3^{a}	27.7±0.2^{a}	2.89±0.02^{a}
90	56.1±2.8^{a}	0.56±0.01^{b}	892±4^{a}	27.8±0.1^{a}	2.86±0.02^{a}
120	54.5±2.3^{a}	0.60±0.01^{a}	882±3^{b}	27.7±0.2^{a}	2.85±0.02^{a}
Solid-to-solvent ratio, g cm^{-3} ** (extraction time 30 min)					
1:10	30.6±1.5^{e}	0.91±0.03^{a}	898±1^{c}	28.1±0.1^{b}	2.77±0.01^{c}
1:20	56.8±1.9^{c}	0.55±0.01^{b}	898±2^{c}	27.7±0.2^{c}	2.89±0.02^{a}
1:30	49.9±2.5^{d}	0.48±0.01^{c}	893±4^{c}	28.3±0.2^{b}	2.62±0.01^{d}
1:40	71.6±3.0^{b}	0.40±0.01^{d}	941±2^{a}	29.0±0.1^{a}	2.84±0.01^{b}
1:50	86.0±2.9^{a}	0.29±0.01^{e}	817±2^{b}	27.2±0.4^{c}	2.42±0.01^{e}

*Values with different letters (a-e) in variable group showed statistically significant differences (p<0.05; n=3; analysis of variance, Duncan's *post-hoc* test); **the solid-to-solvent ratio represents the mass of plant material per volume of the extraction solvent

It can be seen in the literature that the extraction yield primarily depends on the extraction time, grind size, used solvent, and temperature [46]. The literature states that the extraction yield is expected to be higher with a prolonged extraction time, since the solvent can penetrate the plant more extensively [47,48], but there are also examples in accordance with our results, that show that the increase in the duration of maceration does not always lead to a higher yield, which can be related to the plant type [49].

On the other hand, the solid-to-solvent ratio significantly affected the extraction yield at the extraction time of 30 min (Table 2). Namely, the highest extraction yield was in the extract prepared using 1 g : 50 cm^3 ratio (86.0±2.9 %), while the lowest was in the extract with a 1 g : 10 cm^3 ratio (30.6±1.5 %). The presented results of the extraction yield did not correlate with the TP yield which can be explained by the fact that the amount of the extracted ballast substances, such are lipids, sugars, and proteins also influenced the value of the extraction yield [50]. The literature states that the extraction yield is expected to increase with a lower solid-to-solvent ratio [51]. This could be explained by the fact that there is an excessive amount of plant material (at a 1 g : 10 cm^3 ratio) [23]. Additionally, a higher amount of the solvent (at a lower solid-to-solvent ratio) prevents the saturation of the extraction medium, and therefore allows prolonged release of the active and non-active compounds.

3. 4. Conductivity, density, surface tension, and viscosity of the extracts

According to Suliman *et al.* [52], conductivity represents an indicator of total dissolved compounds, as well as a predictor of the antioxidant potential of a sample. The conductivity of *S. montana* extracts significantly differs only between some samples prepared at various extraction times, but without a noticeable trend due to quite low conductivity values (0.55 to 0.61 mS cm^{-1}), with some minor differences (Table 2). On the other hand, conductivity continuously decreased with the decrease in the solid-to-solvent ratio (from 0.91 to 0.29 mS cm^{-1}). According to the literature data [53], the number of ions per unit volume and their drift velocity affect the electrical conductivity of a liquid. Drift velocity of an ion depends on the strength of the electric field, the ion mass, temperature of the solution, as well as on other variables. Thus, the electrical conductivity of various liquids may have a wide range of values. One study [54] reported that plant extracts with lower conductivities showed lower antioxidant capacities. Nevertheless, this was not the case with *S. montana* ethanol extracts in the present study. The reason is probably related to different compounds in the extracts that can affect the conductivity but do not possess antioxidant potential.

Since the molecules in liquids are loosely packed, several parameters can affect the density of liquids, the most important being temperature along with the composition. As can be seen in Table 2, density of *S. montana* extracts varied from 875 to 941 kg m^{-3}, whereas the surface tension varied from 27.4 to 29.0 mN m^{-1}. However, the highest density (among the extracts obtained at different extraction times) was determined after 60 and 90 min of maceration (898±3 and 892±4 kg m^{-3}, respectively), while the extracts obtained at other extraction times had statistically significantly lower density. The extract prepared at a 1 g : 40 cm^3 ratio had the highest density (941±2 kg m^{-3}), while the extract obtained at a 1 g :50 cm^3 ratio showed the lowest density (817±2 kg m^{-3}). According to the literature [55], density correlated to the extraction yield, which was the case with some of the *S. montana* ethanol extracts obtained at different extraction times, where the density is correlated to the TPC yield as well. But in the case of *S. montana* extracts prepared at different solid-to-solvent ratios, it was the opposite (Table 2). However, this finding can be explained by the fact that the amount of the extraction solvent also influences the density of the extracts, thus lower solid-to-solvent ratio causes lower density regardless of the extraction yield and polyphenol content.

Ability of a liquid surface to operate like a stretched elastic membrane is known as surface tension. The fundamental factors affecting this physical characteristic are the forces of attraction between the particles at the interface of the liquid and gas, solid, or another liquid [56]. Surface tension did not differ significantly between the extracts obtained at different extraction times (Table 2). On the other hand, the extract obtained at the 1 g : 40 cm^3 ratio exhibited statistically significantly higher surface tension (29.0±0.1 mN m^{-1}) in comparison to the extracts obtained at other ratios, where the lowest values of surface tension were measured in extracts obtained at 1 g : 20 cm^3 and 1 g : 50 cm^3 ratios (27.7±0.2 and 27.2±0.4 mN m^{-1}, respectively). Higher surface tension is related to the relatively high interaction between water molecules by hydrogen bonds that can cause a decrease in polyphenol diffusion [57]. However, this is in

agreement only with the results of flavonoid content obtained at 1 g : 20 cm^3 and 1 g : 30 cm^3 ratios (Table 1), where *S. montana* extracts that exhibited lower surface tensions (Table 2) contained higher flavonoid amounts.

Viscosity is the measure of a liquid's resistance to flow. Viscosity often varies with the temperature generally decreasing as the temperature is increased. The viscosity of *S. montana* extracts obtained at different extraction times did not differ statistically, and values varied from 2.85 to 2.90 mPa·s. However, as was expected, the viscosity of the extracts prepared using different solid-to-solvent ratios differed significantly. Namely, the extract obtained using the 1 g : 20 cm^3 ratio has shown the highest viscosity (2.89±0.02 mPa·s), while the extract prepared at the 1 g : 50 cm^3 ratio had the lowest viscosity (2.42±0.01 mPa·s). However, it was not the case with *S. montana* ethanol extracts, where probably hydrolysis of various compounds in ethanol extracts (with higher solid to solvent ratio) resulted in a lower viscosity [58]. Namely, according to the literature data [59,60], polyphenol compounds are susceptible to degradation (hydrolytic and oxidative reactions), particularly in the surrounding water (also presented in our 50 % ethanol extracts).

3. 5. Potential applications of *Satureja montana* extracts

The present study provides information on the physicochemical properties and antioxidant potential of *S. montana* extracts. Due to the long-term use of *S. montana* as an antioxidant, anti-inflammatory, and antimicrobial agent, the obtained extracts can be implemented in dermal pharmaceutical and cosmetic formulations. However, additional investigations in terms of the antimicrobial and anti-inflammatory activities of the extracts should be performed. Furthermore, due to the diuretic, antidiarrheal, antispasmodic, anticholinesterase, and cytotoxic effects of *S. montana*, the incorporation of its extracts in food, functional food, and *per os* pharmaceutical products can be examined. In that case, the encapsulation of the extracts is necessary with the aim to improve the stability and bioavailability of the polyphenols.

4. CONCLUSION

The aim of this work was to determine the optimal extraction time and solid-to-solvent ratio for obtaining extracts from the plant material of cultivated, rare chemotype of *S. montana* (ct. thymol), with the highest total polyphenol and flavonoid contents, antioxidant activities, as well as satisfactory physical characteristics. In general, the amount of polyphenolic compounds increased with the lower solid-to-solvent ratio, and it was also the highest for the extraction period of 60 min. On the other hand, antioxidant potential in FRAP and CUPRAC assays reached the highest value after 15 min of maceration, while the highest $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ neutralization capacity was obtained after a 30 min period. Additionally, antioxidant activity determined in ABTS, FRAP, and CUPRAC assays had the highest values for a solid-to-solvent ratio of 1 g : 50 cm^3 , while IC_{50} in the DPPH assay was the lowest (the highest $DPPH^{\bullet}$ radical scavenging potential) for extracts obtained at a ratio of 1 g : 30 cm^3 . Physical characteristics of the extracts did not statistically differ when the extraction time was the observed variable. However, the density and surface tension were the highest in the extract obtained at the 1 g : 40 cm^3 ratio, and the highest extract conductivity was acquired in the extract obtained at the 1 g : 10 cm^3 ratio. The extraction yield increased with the decrease in the solid-to-solvent ratio, thus being the highest for the 1 g : 50 cm^3 ratio, and the highest viscosity was found in the extract obtained at the 1 g : 20 cm^3 ratio. In conclusion, the optimized conditions for maceration process from *S. montana* depend on the future application of the extract. Future perspectives with regard to particular plant material may include its incorporation into a certain type of carrier, in order to acquire a product that might be further used in the pharmaceutical, food, or cosmetic industries.

5. NOMENCLATURE

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
PCS	photon correlation spectroscopy	TE	Trolox equivalent
TFC	total flavonoid content	TPC	total polyphenol content

Acknowledgement: *The authors acknowledge their gratitude to the Ministry of Education, Science and Technological Development of Serbia, contract numbers 451-03-68/2022-14/200003, 451-03-68/2022-14/200287, 451-03-68/2022-14/200135, and 451-03-68/2022-14/200019.*

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Optimizacija procesa ekstrakcije iz biljke *Satureja montana* l.: fizičkohemijska karakterizacija ekstrakata

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(Naučni rad)

Izvod

Prikazana studija je imala za cilj da optimizuje ekstrakciju polifenola iz biljke *Satureja montana* L. sa oglednog polja Instituta za proučavanje lekovitog bilja „Dr Josif Pančić", Srbija, variranjem najvažnijih parametara maceracije, odnosa biljnog materijala i rastvarača, i ekstrakcionog vremena. Dobijeni ekstrakti su okarakterisani u pogledu ukupnog sadržaja polifenola (*engl.* total total polyphenol content, TPC), ukupnog sadržaja flavonoida (*engl.* total flavonoid content, TFC), antioksidativnog potencijala, prinosa ekstrakcije, provodljivosti, gustine, površinskog napona i viskoznosti. Vrednosti ukupnog sadržaja polifenola (*engl.* Total Polyphenol Content - TPC) i ukupnog sadržaja flavonoida (*engl.*Total Flavonoid Content - TFC) su bile u opsezima 7,2-92,2 mg ekvivalenta galne kiseline (*engl.* gallic acid equivalent GAE) / g i 3,7-10,9 mg ekvivalenta katehina (*engl.* catechin equivalent CE)/g, redom. Najveće antioksidativne aktivnosti dobijene su u ekstraktima pripremljenim u odnosu biljnog materijala i rastvarača 1 g : 50 cm^{-3}. Najveći prinos ekstrakcije takođe je dobijen u tom ekstraktu (86,0%), dok vreme ekstrakcije nije imalo značajan uticaj. Najveća provodljivost izmerena je u ekstraktu pripremljenom u odnosu biljnog materijala i rastvarača 1 g :10 cm^{-3}. Najveća gustina i površinski napon su bili u ekstraktu pripremljenom u odnosu 1 g : 40 cm^{-3} (941 kg m^{-3} i 29,0 mN m^{-1}, redom). Najveća viskoznost je izmerena u ekstraktu pripremljenom u odnosu 1 g : 20 cm^{-3} (2,89 mPa·s). Naša studija pokazuje mogućnosti za proizvodnju polifenolima bogatog ekstrakta biljke *S. montana* koji se može koristiti u farmaceutskim, prehrambenim ili kozmetičkim proizvodima..

Ključne reči: antioksidativna aktivnost; flavonoidi; izolacija; polifenoli; planinski čubar