Ultrasound-assisted extraction of polyphenols from *Thymus serpyllum* and its antioxidant activity

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Abstract

The present study was designed to establish and optimize a method for extracting natural bioactive compounds from *Thymus serpyllum* which possess antioxidant, antimicrobial, antispasmodic and stimulant properties. Ultrasound-assisted extraction (UAE) is a well-established method in the processing of plant material, particularly for extraction of bioactive substances such as polyphenols. The influential factors including extraction time (3, 7 and 10 min), solid:solvent ratio (1:10, 1:20 and 1:30) and particle size (0.3, 0.7 and 1.5 mm), have been studied to optimize the extraction process, while using 30% ethanol as an extraction medium and amplitude set to 65%. The yield of UAE was expressed *via* total phenol content and antioxidant activity of the obtained extracts. The optimum process parameters were found to be: extraction time, 3 min; solid:solvent ratio, 1:30; particle size, 0.3 mm. Under these conditions, the yield of total polyphenols was raised up to 23.03 mg/L GA and the highest antioxidant activity was recorded (10.32 mmol/mg Trolox and IC_{50} of 3.00 mg/ml).

Keywords: *Thymus serpyllum*, ultrasound-assisted extraction, polyphenols, antioxidant activity.

Available online at the Journal website: http://www.ache.org.rs/HI/

SCIENTIFIC PAPER

UDC 635.71:66.061:615:54

Hem. Ind. **70** (4) 391–398 (2016)

doi: 10.2298/HEMIND150629044J

Lamiaceae family represents an important component of Mediterranean shrub vegetation, especially in dry and arid environments. *Thymus* species grow wild in the Mediterranean environment and there are several ecotypes which are different in morphology and highly chemically polymorphic [1,2]. The number of species within this genus is depending on taxonomical point of view and usually assumed to be larger than 200 [3]. The diversity of biological activities of these plants may be a consequence of their rich chemical diversity. *Thymus serpyllum*, well-known as wild thyme is perennial, herbaceous plant of genus *Thymus* and possesses aromatic, antiseptic, analgesic, diuretic, diaphoretic, carminative, antioxidant, spasmolytic, anti-inflammatory and stimulant properties. Also, it has been used in mouth washes and gargles and against cough and cold because of antimicrobial activities of its extracts [4]. The main components of *T. serpyllum* are essential oils (44.4% carvacrol, 14% *o*-cymene, 6.47% α-terpineol, 6.06% α-pinene and 5.25% β-caryophyllene), phenolic acids (mainly rosmarinic, caffeic and chlorogenic acid) and flavonoids (naringenin, dihydroquercetin, apigenin, eriodictyol, quercetin and rutin). These secondary metabolites play important biological roles and possess pharmacological effects including antioxidant and anticarcinogenic activities, protection against coronary diseases (prevention of atherosclerosis, antiarrhythmic and antihypertensive effect), lipid lowering activity, effect on central nervous system, antimicrobial, anti-inflammatory and analgesic activities [4–6]. Content of polyphenolics and their structure (the number and position of the hydroxyl groups in a molecule) significantly influence the pharmacological properties of medicinal plants. Water, methanol and ethanol are the most frequently used solvents for extraction of polyphenolics from plant material. One of the frequently applied techniques for extraction of active compounds is extraction in the Soxhlet apparatus. Due to the relatively long extraction time and to the considerable amounts of the samples and consumed solvents, this technique tends to be replaced by more modern extraction methods [7]. In this study, dry flowering aerial part of *T. serpyllum* (*Serpylli herba*) was evaluated as a source of polyphenolic compounds, which are extracted by the application of ultrasound probe instead of the traditional ways of extraction (maceration, percolation, Soxhlet). Ultrasound extraction has become a good alternative extraction method when compared to classical methods due to its high efficiency, low energy, shortening of extraction time and solvent consumption. Ultrasound assisted extraction is a well-established method in the processing of

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Paper received: 29 June, 2015
Paper accepted: 24 July, 2015

plant material, particularly for extraction of bioactive substances such as polyphenols [8]. The ultrasound waves in the extraction medium induce mechanical, cavitation and thermal effects that can lead to the disruption of cell walls, without causing significant changes in the structural and functional properties of the most target compounds [9]. Optimization of the extraction has been carried out through varying time of extraction, solid:solvent ratio and particle size.

In this paper, the extraction conditions for extracting polyphenols from *Serpylli herba* by using ultrasonic probe have been studied and optimum conditions for ultrasound-assisted extraction were established. Extraction efficiency was expressed *via* total polyphenols content (Folin–Ciocalteu method) and antioxidant activity (ABTS and DPPH methods) of the obtained extracts.

MATERIALS AND METHODS

Plant materials and reagents

In order to optimize the extraction method, air-dried herbs of wild thyme (*T. serpyllum*) were commercially purchased from the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia. These commercial samples differ in particle size (0.3, 0.7 and 1.5 mm).

The following reagents of the analytical purity grade were used: ethanol (Fisher Science, UK), Folin–Ciocalteu reagent (Merck, Darmstadt, Germany), sodium carbonate (Fisher Science, UK), gallic acid (Merck, Darmstadt, Germany), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS (Sigma–Aldrich, St. Louis, MO, USA), potassium persulfate (Centrohem, Belgrade, Serbia), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or Trolox (Sigma–Aldrich, St. Louis, MO, USA) and 2,2-diphenyl-1-picrylhydrazyl or DPPH (Sigma-Aldrich, St. Louis, MO, USA).

Ultrasound-assisted extraction

Ultrasound-assisted extractions were performed by using ultrasound probe (Bandelin, Ultrasonic Homogenizer HD 2200, processor of 200 W output with a 20 kHz converter and a solid titanium probe of 13 mm). Amplitude applied for the extraction was set to 65%. Samples for ultrasound probe treatment were placed in 250 mL beaker and mixed with appropriate solvent (30% ethanol) in different solid:solvent ratio (1:10, 1:20 and 1:30). Larger volume of extraction mixture was applied because of technical properties of the probe. Samples were treated for 3, 7 and 10 min with power ultrasound, high intensity and low frequency at room temperature. After extraction, the obtained extracts were filtered through a 0.45 μm cellulose filter and reconstituted filtrates were properly diluted up to a required concentration for further analysis.

Determination of total polyphenolic content

Total polyphenolic content in ethanol extracts was determined by the Folin–Ciocalteu (FC) procedure. A volume of 6 mL water and 100 μL properly diluted sample was mixed with 500 μL of FC reagent previously diluted with distilled water in a 1:2 ratio. After that, 1.5 mL of 20% sodium carbonate solution was added and the volume was made up to 10 mL. The samples were shaken and left in the dark for 2 h to react. Then the absorbance of blue coloration was measured at 765 nm against a blank using the Shimadzu UV spectrophotometer UV-1800. The blank contained water, 30% ethanol, FC reagent and sodium carbonate solution in the same ratio as in the samples. All samples were done in triplicate. The same procedure was done with gallic acid standard (concentrations of 100, 200, 300, 400, 500, 600 and 700 mg/L) and a calibration curve was calculated. The total polyphenolic content (TPC) was expressed as milligram per litre of gallic acid equivalents (mg/L GAE) [10].

Determination of free radical-scavenging ability

ABTS method

The $ABTS^{\bullet+}$ scavenging assay was based on the procedure described by Re *et al*. with a slight modification [11]. $ABTS^{\bullet+}$ was produced by mixing 5 ml of ABTS water stock solution (7 mM) with 88 μL of potassium persulfate (140 mM). Before use, the mixture was incubated in a fridge in the dark for 16–20 h to prepare ABTS radical cation ($ABTS^{\bullet+}$). Freshly-prepared $ABTS^{\bullet+}$ working solution ($ABTS^{\bullet+}$ stock solution diluted with ethanol to achieve an absorbance of 0.70±0.02 at 734 nm) was used. To a volume of 2 ml of $ABTS^{\bullet+}$ working solution, 20 μL of diluted extract were added (100 μL of ethanol extract and 900 μL of solvent) and, after 6 min of incubation in the dark, the absorbance was measured at 734 nm. The blank contained 2 mL of ethanol and 20 μL of solvent. The scavenging capacity was calculated as $\Delta A = A_0 - A_x$ (where A_0 refers to the absorbance of 2 mL $ABTS^{\bullet+}$ working solution and 20 μL of solvent; A_x is the absorbance of sample). All measurements were performed in triplicate. The same procedure was done with Trolox standard (concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mM), and a calibration curve was calculated. The antioxidant activity was expressed as mmol per mg of Trolox (mmol/mg Trolox).

DPPH method

This method is based on the reduction of stable DPPH radical by antioxidants present in tested extracts. In the presence of antioxidants purple color of the DPPH radical solution changes to a bright yellow and the intensity of this change can be monitored spectro-

photometrically [12]. Briefly, DPPH was diluted in ethanol to achieve an absorbance of 0.8 at 517 nm. Ethanol extracts were further diluted in ethanol to obtain concentration 5–160 μL/mL. After that, 200 μL of diluted extract was added to 2.8 mL of DPPH solution. The mixture was kept in dark for 20 min and the absorbance at 517 was measured. The blank contained 2.8 mL of ethanol and 200 μL of solvent. The scavenging IC_{50} = = $100(A_0 - A_x)/A_0$ capacity was calculated as (where A_0 refers to the absorbance of 2.8 mL DPPH working solution and 200 μL of solvent; A_x is the absorbance of sample). All determinations were performed in triplicate. Values of IC_{50} were calculated from the regression equation, prepared from the concentration of samples and percentage inhibition of DPPH. Results were expressed as IC_{50} (mg/mL), defined as the concentration of extract required to scavenge 50% of free radicals.

Statistical analysis

All data were subjected to analysis of variance (ANOVA), significant differences among mean values from triplicate analysis ($p < 0.05$) were determined by Duncan's multiple range tests with data analysis statistical tool of Statistica 7.0.

RESULTS

In order to study the effects of different extraction conditions of ultrasound-assisted extraction, three different particle size (0.3, 0.7 and 1.5 mm) were tested in this study in combination with different extraction time (3, 7 and 10 min) and solid:solvent ratio (1:10, 1:20 and 1:30). The obtained extracts were first tested for total polyphenols content by means of spectrophotometric analysis.

The highest content of polyphenols (23.03 mg/L GAE) was determined in the extract obtained at a particle size of 0.3 mm and solid:solvent ratio 1:30 after 3 min of extraction. The lowest total polyphenols content was detected in extracts obtained with a particle size of 1.5 mm and at solid:solvent ratio 1:10, after 3 and 10 min of the extraction (12.07–12.10 mg/L GAE). Statistical analysis by means of analysis of variance (ANOVA) confirmed a significant influence of solid:solvent ratio on the content of extracted phenolic compounds ($p < 0.05$). The influence of the particle size and extraction time did not prove to be statistically significant. The effects of extraction time and solid:solvent ratio on total polyphenols content is illustrated in 3D surface plot (Figure 1). As seen in Figure 1, TPC increases with increase in solid:solvent ratio, regardless extraction time. Figure 2 shows the effects of extraction time and particle size on TP and reveals the best conditions of extraction: particle size 0.2–0.4 mm and extraction time 2–4 min. In Figure 3, the surface plot shows the effect of solid:solvent ratio and particle size on TP. In the region of solid:solvent ratio 1:28–1:32 and particle size between 0.2 and 0.6 mm, the highest values of total polyphenols are recorded. As seen in Figure 3, there is no significant difference between the different particle sizes in total polyphenols content.

In the Tables 1 and 2 the results of two antioxidant activity assays (ABTS and DPPH method) are presented. Since these two assays are based on different reactions ($ABTS^{\bullet+}$ + any reducing agent *X* and DPPH + any molecule with a weak *X*–H bond), it is possible to obtain different results for the antioxidant capacity of the same samples and a better insight in their properties. The main limitations of these tests are that the probes are chemically very different from the radicals respon-

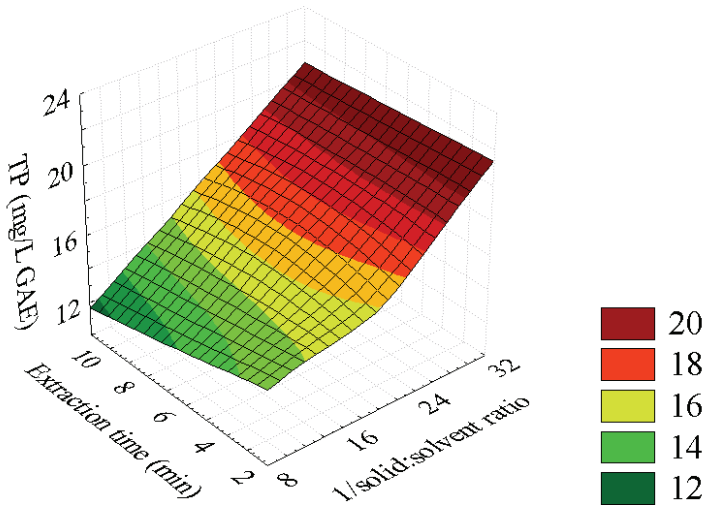

Figure 1. Effect of extraction time and solid:solvent ratio on total polyphenols content (mg/L GAE) of T.serpyllum. The value of the missing independent variable in the plot was kept at the centre point.

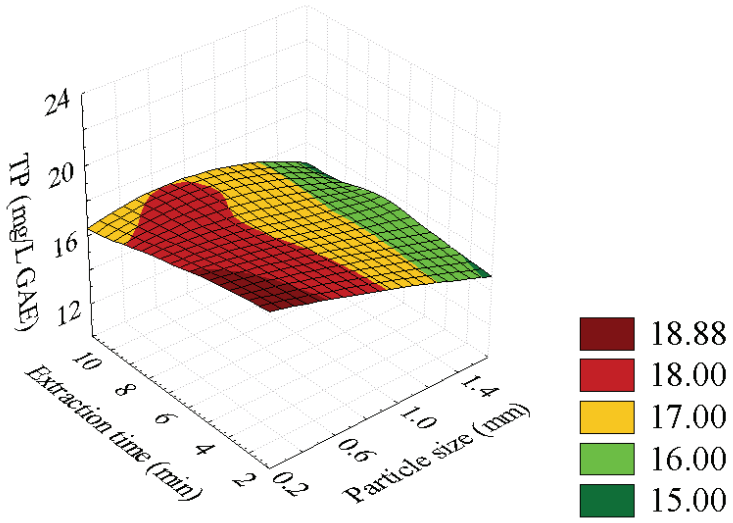

Figure 2. Effect of extraction time and particle size on total polyphenols content (mg/L GAE) of T.serpyllum. The value of the missing independent variable in the plot was kept at the centre point.

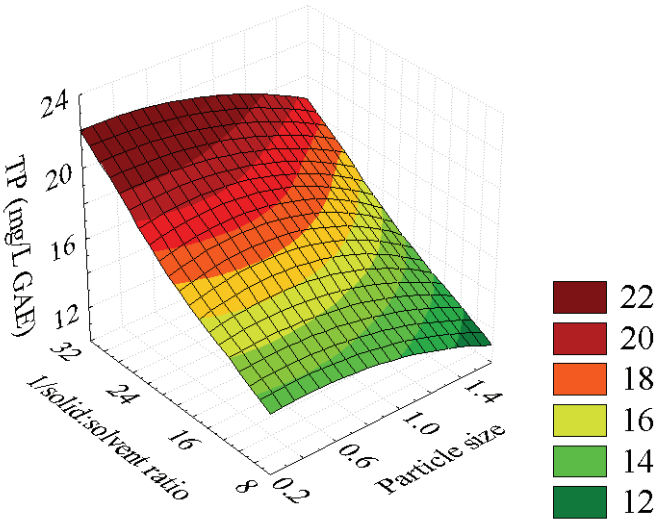

Figure 3. Effect of solid:solvent ratio and particle size on total polyphenols content (mg/L GAE) of T. serpyllum. The value of the missing independent variable in the plot was kept at the centre point.

Table 1. Antioxidant activity of T.serpyllum ethanol extracts – ABTS method (mmol/mg Trolox)

Particle size, mm	Extraction time, min	Solid:solvent ratio		
		1:10	1:20	1:30
0.3	3	7.53±0.04	8.47±0.88	10.32±0.12
	7	6.22±0.04	9.51±0.53	10.05±0.36
	10	5.72±0.03	9.88±0.27	9.19±0.32
0.7	3	7.92±0.39	8.74±0.56	9.56±0.56
	7	6.28±0.27	9.00±0.23	9.93±0.24
	10	5.83±0.05	7.76±0.08	9.11±0.72
1.5	3	7.43±0.85	8.39±0.11	10.00±0.40
	7	6.20±0.05	8.74±1.23	9.94±0.01
	10	6.39±0.25	8.57±0.03	9.22±0.02

Table 2. Antioxidant activity of T.serpyllum ethanol extracts – DPPH method (IC_{50} / mg mL^{-1})

Particle size, mm	Extraction time, min	Solid:solvent ratio		
		1:10	1:20	1:30
0.3	3	3.37±0.04	3.05±0.01	3.00±0.02
	7	3.67±0.07	3.04±0.00	3.17±0.06
	10	4.53±0.13	3.11±0.07	3.17±0.01
0.7	3	3.71±0.19	3.51±0.16	3.40±0.14
	7	4.70±0.12	3.39±0.13	3.22±0.01
	10	4.66±0.17	3.68±0.18	3.05±0.02
1.5	3	4.78±0.55	3.55±0.11	3.3±00.10
	7	4.71±0.25	3.78±0.23	3.44±0.11
	10	4.62±0.16	3.66±0.03	3.32±0.08

sible for the oxidation in *in vivo* conditions. These results indicate a "radical trapping power" rather than true antioxidant activity. This means that molecules able to scavenge these synthetic radicals are not necessarily able to stop the oxidative chain. However, due to the similar electronic configuration between DPPH and peroxyl radicals, the significance of this method could be greatly improved under appropriate settings, particularly by monitoring the entire time evolution of the reaction, instead of performing single-point measurements [13,14].

The highest value of antioxidant capacity in the ABTS assay was recorded after 3 min of extraction, with particle size of 0.3 mm and at solid:solvent ratio 1:30, whereas the lowest antioxidant activities were detected after 10 min of extraction with solid:solvent ratio 1:10 (10.32 mmol/mg Trolox and 5.72–5.83 mmol/mg Trolox, respectively). The best results of antioxidant capacity in DPPH assay (the lowest values of IC_{50}) were determined in extracts obtained with a particle size of 0.3 mm and solid:solvent ratio 1:20 and 1:30 (IC_{50} 3.00––3.17 mg/mL). Therefore, the lowest values of antioxidant activity (very high values of IC_{50}) were detected with solid:solvent ratio 1:10 and particle size of 1.5 mm (IC_{50} 4.62–4.78 mg/mL).

DISCUSSION

Effect of extraction time

Fick's second law predicts how diffusion causes the concentration to change with time and prolonged time of extraction is expected to increase the content of soluble polyphenols in the extracts. Furthermore, the duration of the extraction is a function of the molecular weight of the active substances and the molecular weight of ballast substances. If the molecular weight of the active substances is smaller than molecular weight of ballast material, extraction process will last shorter, since the active ingredients diffuse rapidly [15]. However, in this paper, the effect of time was not statistically significant and an excessive time was not useful for better extraction of polyphenols. A possible explanation lies behind sensitivity of phenolic compounds especially at higher temperatures [16]. Similarly, extraction time did not prove to be statistically significant in case of ultrasound-assisted extraction of polyphenols from *Urtica dioica* [17], neither from *Lawsonia inermis* [18].

Polyphenols are widely seen as very unstable and highly susceptible to degradation. The stability of polyphenols under different conditions is a very important aspect which has to be taken into account to ensure that polyphenolic compounds have the desired properties and maintain their activity and structure during the different stages of processing, which can involve high temperature, light, oxygen, solvents, the presence of enzymes, proteins and metallic ions [19]. Therefore, shorter extraction time (3 min) is more acceptable both in terms of yield of bioactive compounds and energy efficiency.

In addition, antioxidant activity slightly decreased with prolonged extraction time and this could be explained by sensitivity of natural antioxidant compounds to heat released by the ultrasound probe and at air exposure. Also, ultrasound is known to generate free radicals, which can degrade natural antioxidants and cause decrease of the antioxidant capacity [20]. Similarly, in case of ethanol extracts of *Yellow tea*, prolonged extraction time caused a slight decrease in antioxidant capacity [21]. The overall conclusion is that shorter time of extraction is more appropriate for *T. serpyllum* extraction too, because of better protection of antioxidants present in the extract and because of energy efficiency.

Effect of the solid:solvent ratio

In general, by extending the extraction time and increasing of solid:solvent ratio, the amount of extractive substances is increased because of higher concentration gradient. Moreover, a larger solvent volume can dissolve constituents more effectively, which leads to

an enhancement of the extraction yield of polyphenols [15,22]. In this study, solid:solvent ratio influenced significantly TP content and the extraction yield of polyphenols increased of the solid:solvent ratio (Figures 1 and 3). This result is consistent with literature data on ultrasound-assisted extraction of rutin and quercetin from *Eunymus alatus* [22]. This could be explained by the better performance of acoustic cavitation in the dilute solution (1:30) than in the more viscous one (1:10). Besides that, the diffusion rate is inversely proportional to the viscosity of the medium and the molecular weight of the active substances [23]. In addition, both assays showed that antioxidant activity significantly decreased with the decreasing ratio drug:solvent, especially when this ratio was 1:10.

Effect of particle size

The transition of active compounds from plant material in the extraction medium is directly proportional to the diffusion coefficient, the contact surface between drug and solvent and concentration gradient. In general, the extraction in a shorter time of a drug which has a higher degree of fragmentation, more effectively recover of the target compounds can be achieved [15]. This can be explained by increase in exchange surface and decrease in path length necessary for the solute to reach the surface, both help to reduce the extraction time. On the other hand, the use of very small particles may lead to technical difficulties (for example, during the filtration) [24]. According to our results (Figures 2 and 3), the values of total polyphenols decreased with increasing fragmentation degree, but these differences were not statistically significant, as determined by analysis of variance (ANOVA). The highest yield was recorded with the smallest particle size (0.3 mm), which confirmed that the convective mass transfer had the dominant influence. The similar results were obtained in case of extraction conditions of *Ginkgo biloba* [25]. This could be explained by the mechanism of ultrasound extraction, which involves the disruption of the cell walls, reduction of the particle size and the intensification of the mass transfer of the cell content to the extraction medium and easier access of the solvent to the plant cells, caused by the collapse of the bubbles produced by cavitation, regardless of the initial particle size. If this collapse is within a biological material, ultrasound can affect these biological materials and tissues on micro- and a macro-scale [26]. For instance, SEM micrograph analysis in the study of ultrasound-assisted extraction of *E. alatus* revealed a large number of ruptures on the surface of plant caused by ultrasonic waves [22]. As reflected in Tables 1 and 2, particle size did not influence significantly antioxidant capacity tested in ABTS and DPPH assays.

CONCLUSION

The study represents optimization of ultrasound-assisted extraction of *T. serpyllum* with 30% ethanol, and amplitude applied for extraction was set to 65% at room temperature. The optimal conditions (extraction time, solid:solvent ratio and particle size) for the UAE were developed by quantitative analysis of the polyphenols by using Folin–Ciocalteu method and antioxidant activity assays, ABTS and DPPH. The best extraction performance was achieved with a particle size of 0.3 mm and solid:solvent ratio 1:30 after 3 min of extraction. Also, analysis of variance (ANOVA) confirmed a significant influence of solid:solvent ratio on the content of extracted phenolic compounds (1:30 > 1:20 > > 1:10), whereas time of extraction and particle size had no statistical significant effect on TP.

Acknowledgements

The authors acknowledge their gratitude to the Ministry of Education, Science and Technological Development of Serbia, project numbers 46010 and 46013.

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IZVOD

ULTRAZVUČNA EKSTRAKCIJA POLIFENOLA IZ *Thymus serpyllum* I NJEGOVA ANTIOKSIDATIVNA AKTIVNOST

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(Naučni rad)

U ovom radu je predstavljena i optimizovana metoda za ekstrakciju prirodnih-biološki aktivnih jedinjenja iz *T. serpyllum*, koja poseduju antioksidativnu, antimikrobnu, spazmolitičnu, antiinflamatornu i stimulativnu aktivnost. Ultrazvučna ekstrakcija se sve više koristi u procesu izolovanja aktivnih principa iz biljnog materijala, posebno u ekstrakciji bioaktivnih supstanci poput polifenola. Osnovne prednosti ultrazvučne ekstrakcije nad klasičnim metodama su visoka efikasnost, kraće vreme ekstrakcije i ušteda rastvarača. Ultrazvučni talasi u medijumu indukuju mehaničke, kavitacione i termalne efekte, koji uzrokuju degradaciju ćelijskog zida, bez promena u strukturnim i funkcionalnim karakteristikama ciljanih jedinjenja. Optimizacija procesa ekstrakcije je vršena kroz variranje vremena ekstrakcije (3, 7 i 10 min), odnosa droga:rastvarač (1:10, 1:20 i 1:30) i stepena usitnjenosti (0,3, 0,7 i 1,5 mm), pri čemu je kao rastvarač korišćen 30% etanol i primenjena je amplituda od 65%. Optimalni uslovi su ispitani korišćenjem kvantitativne spektrofotometrijske metode sa Folin-Ciocalteu reagensom. Osim toga, efikasnost ekstrakcije je izražena preko antioksidativne aktivnosti određene u ABTS i DPPH metodama. Utvrđeno je da su najbolji uslovi za ekstrakciju stepen usitnjenosti 0,3 mm, vreme ekstrakcije 3 min i odnos droga:rastvarač 1:30. Pod ovim uslovima prinos ukupnih polifenola je iznosio 23,03 mg/L GA, dok je zabeležena antioksidativna aktivnost bila 10,32 mmol/mg Trolox i IC_{50} 3,00 mg/mL. Nakon statističke analize pokazano je da odnos droga:rastvarač statistički značajno utiče na sadržaj ukupnih polifenola (1:30 > 1:20 > 1:10), dok vreme ekstrakcije i stepen usitnjenosti ne pokazuju statistički značajan uticaj na vrednost ukupnih polifenola. Može se zaključiti da je ekstrakcija ultrazvučnom sondom metod izbora za ekstrakciju polifenola iz *Serpylli herba*, jer je potrebno kraće ekstrakciono vreme, kao i zbog uštede energije, visoke efikasnosti i visokog prinosa ekstrakcije.

Ključne reči: *Thymus serpyllum* • Ultrazvučna ekstrakcija • Polifenoli • Antioksidativna aktivnost