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Free radicals' scavenging capacity of *Thymus serpyllum* L. extracts depending on applied extraction conditions and extraction techniques

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Abstract

Polyphenols, as plant secondary metabolites, achieve strong antioxidant capacity by scavenging free radicals, chelating metals, and interacting with lipid membranes, proteins and nucleic acids. The aim of the present study was the examination of the applied extraction conditions (particle size of plant material, solvent-to-solid ratio, solvent type, and extraction time) and extraction techniques (maceration, heat- and ultrasound-assisted extractions) on the free radicals' scavenging capacity of Thymus serpyllum polyphenol extracts, determined in ABTS and DPPH methods. According to the results, smaller particle size of herbal drug resulted with better antioxidant potential. However, this effect was statistically significant for heat- and ultrasound-assisted extractions according to ABTS assay, and for maceration and heat-assisted extraction according to DPPH assay. Furthermore, the increase in solvent-to-solid ratio from 10:1 to 30:1 resulted in the extracts with higher antioxidant activity regardless of the type of extraction technique, whereas the scavenging activity of ethanol extracts was higher in comparison to aqueous extracts. In terms of extraction time for achieving the highest free radicals' scavenging capacity, 30 min can be recommended for maceration and heat-assisted extraction, and 5 min for processing by ultrasonic probe. In neutralization of ABTS radicals, there were no statistically significant differences between antioxidant capacity of T. serpyllum extracts obtained by using different extraction techniques, whereas in DPPH scavenging activity, the extracts obtained in ultrasound-assisted extraction have shown statistically significantly higher activity. Due to the free radicals' scavenging properties shown in the present study, T. serpyllum extracts may be used in the formulations in food, pharmaceutical, cosmetic, and chemical industries.

Key words: *extraction; polyphenols; scavenging capacity; Thymus serpyllum.*

INTRODUCTION

Natural plant products are widely used in the production of drugs, dietary supplements and functional foods, which in addition to nutritional properties also exhibit pharmacological effects and have great importance in the prevention of diseases. Therefore, the examination of biological activities and chemical characterization of plant species is of exceptional scientific and practical importance, because it represents an essential step in obtaining potent, pharmacologically active natural products [1-3]. By examining the chemical composition and biological activities of natural products used in traditional medicine, various pharmacologically active substances have been identified and isolated, which are still used in pharmacotherapy today.

Oxidation is an essential reaction that provides energy for most biological processes in a living being. On the other hand, the oxidation reaction is one of the most important pathways for the formation of free radicals in food, drugs and organisms as well [4]. Although the human body uses an innate defense mechanism against free radicals, the presence of pathogens, pollution, chemicals, stress, and pathological changes formed large amounts of free radicals in living organ-

isms, which exceed the capacity of cellular defense. Thus, free radicals cause irreversible damage of lipids, proteins and nucleic acids, and consequently death of cells [5].

Antioxidants are compounds that have the ability to slow or inhibit the oxidation of lipids, proteins, and other molecules, preventing the initiation or propagation of oxidation chain reactions [4,6]. Namely, plants synthesize a large number of antioxidant components in different concentrations and with different physicochemical characteristics. Polyphenol compounds can prevent free radical damage due to their structure and reducing potential, which give them a special role in the adsorption and neutralization of superoxide anions, nitrogen oxides, peroxides, and other free radicals. Polyphenols achieve strong antioxidant capacity by scavenging free radicals, chelating metals, and interacting with lipid membranes, proteins and nucleic acids [7,8].

Several studies have shown strong antioxidant activity of *Thymus serpyllum* (wild thyme) essential oil [2,9]. In addition, herbal products of *T. serpyllum* inhibited copper-induced oxidation of low-density lipoproteins [10]. Petrović *et al*. [11] have shown that the essential oil obtained from wild thyme herb by hydrodistillation process possessed a significantly better ability to neutralize DPPH radicals, compared to the synthetic antioxidants butylhydroxyanisole and butylhydroxytoluene. According to the literature, in comparison to other *Thymus* species, wild thyme essential oil showed the best antioxidant activity in neutralizing free DPPH radicals, the highest reduction potential in the transformation of Fe^{3+} to Fe^{2+} and the highest inhibition of β-Carotene oxidative degradation [2]. However, the influence of different extraction conditions and extraction procedures on antioxidant potential of wild thyme extracts has not been yet scientifically documented.

The aim of the presented study was the investigation of the influence of various extraction conditions (1) particle size of plant material (0.3, 0.7, and 1.5 mm), (2) solvent-to-solid ratio (10:1, 20:1, and 30:1), (3) solvent type (water, 30, 50, and 70% ethanol), and (4) extraction time (depending on the applied extraction procedure), and different extraction techniques (maceration, heat- and ultrasound-assisted extractions) on the free radicals' scavenging capacity of liquid *T. serpyllum* extracts.

MATERIALS AND METHODS

Plant material and reagents

T. serpyllum herb was obtained from the Institute for Medicinal Plants Research ''Dr Josif Pancic", Belgrade, Serbia. The following chemicals were used: ethanol (Fisher Scientific, UK), potassium persulfate (Centrohem, Serbia), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) - ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid - Trolox and 2,2-diphenyl-1-picrylhydrazyl - DPPH (Sigma-Aldrich, USA).

Extraction procedures

Maceration was performed on Unimax 1010 air shaker (Heidolph, Germany) at 25 °C using three particle sizes (0.3, 0.7, and 1.5 mm), three solvent-to-solid ratios (10:1, 20:1, and 30:1), four solvent types (water, 30, 50, and 70% ethanol), and five extraction times (5, 15, 30, 60, and 90 min). Heat-assisted extraction was carried out in the incubator shaker KS 4000i control (IKA, Germany) at 80 °C using the same levels of particle size, solvent-to-solid ratios, and solvent types as in the case of maceration and three extraction times (5, 15, and 30 min). In ultrasound-assisted extraction, the ultrasound processor (750 W, Sonics, USA) with a converter (20 kHz) and a solid titanium probe (diameter of 19 mm) were used at amplitude of 80%, under the same factor levels as in the case of heat-assisted extraction.

DETERMINATION OF FREE RADICALS' SCAVENGING CAPACITY

ABTS assay

The antioxidant activity of liquid wild thyme extracts was analyzed according to ABTS assay previously published by Re *et al*. [12]. The method is based on the measurement of the absorbance decrease (734 nm, UV Spectrophotometer UV-1800, Shimadzu, Japan), caused by the reduction of free ABTS radicals by polyphenols. A mixture of ABTS solution and 88 μL of potassium persulfate solution was left to react for 24 h in a fridge. Subsequently, a mixture was diluted with ethanol (absorbance of 0.700 ± 0.020) and diluted liquid wild thyme extract was added in ABTS mixture. The antioxidant capacity was calculated using the equation: $\Delta A = A_0 - Ax$ (A_0 - the absorbance of control, A_x – the absorbance of the sample). Antioxidant potential was expressed as mmol Trolox equivalents per milliliter of extract (mmol Trolox/mL).

DPPH ASSAY

Furthermore, the antioxidant potential of liquid *T. serpyllum* extracts was analyzed according to DPPH assay previously published by Horžić *et al*. [1]. The test is based on the measurement of the absorbance decrease at 517 nm, caused by the neutralization of free DPPH radicals by polyphenol compounds. Ethanol DPPH solution (absorbance ~0.800) was mixed with different concentrations of liquid wild thyme extract. The antioxidant activity was calculated as follows:

$IC_{50} = (A_0 - Ax) \cdot \frac{100}{A_0}$ (A_0 – the absorbance of control,

A_x – the absorbance of the sample). Antioxidant potential was expressed as IC_{50} (mg/mL), i.e. the concentration of extract required to neutralize 50% of DPPH radicals.

Statistical analysis

In the present study, all extraction procedures, measurements and analyses were done in triplicate. The statistical analysis was performed by using analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test within the statistical software, STATISTICA 7.0. The differences were considered statistically significant at $p < 0.05$. The data are presented as mean ± standard deviation. Independent variables were particle size, solvent-to-solid ratio, solvent type, extraction time, and extraction technique, whereas antioxidant potential (obtained in ABTS and DPPH assays) was dependent variable.

RESULTS AND DISCUSSION

The impact of various particle sizes (0.3, 0.7, and 1.5 mm), solvent-to-solid ratio (10:1, 20:1, and 30:1), solvent types (water, 30, 50, and 70% ethanol), extraction times (5-90 min for maceration and 5-30 min for heat- and ultrasound-assisted extractions), and extraction techniques on the antioxidant potential of liquid wild thyme extracts was examined (ABTS and DPPH assays). Considering that these assays are based on different principles and reactions, the results obtained thereof may provide good insight into overall free radicals' scavenging capacity of the extracts. The results are shown in **Table 1** (for extraction conditions) and in **Figure 1** (for extraction techniques).

Regarding the impact of particle size, higher degree of herbal drug disintegration resulted in better antioxidant activity, but this effect was statistically significant for heat- and ultrasound-assisted extractions according to ABTS assay, and for maceration and heat-assisted extraction according to DPPH assay (**Table 1**).

Table 1. The influence of different extraction conditions on the antioxidant activity of liquid *Thymus serpyllum* extracts obtained using maceration, heat- and ultrasound-assisted extractions (ABTS and DPPH assays).

		Antioxidant activity-ABTS [mmol Trolox/mL]±SD			**Antioxidant activity-DPPH IC_{50} [mg/mL]±SD**		
		Extraction techniques			Extraction techniques		
Factor	Level	**M**	**HAE**	**UAE**	**M**	**HAE**	**UAE**
Particle size [mm]	0.3	10.5±0.5^{a}	11.9±0.8^{a}	11.3±0.1^{a}	3.4±0.1^{a}	3.0±0.3^{a}	2.7±0.2^{a}
	0.7	10.4±0.6^{a}	11.0±0.3^{a}	10.1±0.4^{b}	3.4±0.2^{a}	3.5±0.5^{ab}	2.8±0.5^{a}
	1.5	10.2±0.6^{a}	9.9±0.6^{b}	8.5±0.5^{c}	4.0±0.1^{b}	3.7±0.1^{b}	2.9±0.4^{a}
Solvent-to-solid ratio	10:1	8.3±0.2^{c}	8.3±0.3^{c}	7.7±0.8^{c}	4.3±0.2^{b}	4.2±0.3^{b}	3.2±0.1^{b}
	20:1	11.2±0.3^{b}	11.1±0.1^{b}	10.5±0.7^{b}	3.3±0.3^{a}	3.3±0.3^{a}	2.7±0.1^{a}
	30:1	13.3±0.9^{a}	13.4±0.4^{a}	11.7±0.1^{a}	3.1±0.4^{a}	2.8±0.6^{a}	2.5±0.3^{a}
Solvent type [% of ethanol]	0	8.2±0.8^{c}	6.8±0.2^{b}	6.8±4.2^{c}	4.3±0.3^{d}	4.5±0.2^{b}	4.7±0.8^{c}
	30	11.4±0.3^{b}	11.9±0.8^{a}	10.3±0.4^{b}	3.7±0.1^{c}	2.5±0.2^{a}	2.4±0.1^{b}
	50	13.0±0.3^{a}	12.8±0.4^{a}	11.7±0.1^{a}	2.7±0.4^{a}	2.3±0.4^{a}	2.0±0.1^{a}
	70	11.1±0.4^{b}	12.1±0.1^{a}	11.0±0.7^{ab}	3.4±0.1^{b}	2.4±0.1^{a}	2.0±0.2^{a}
Time [min]	5	8.5±0.2^{c}	10.2±0.2^{b}	9.7±0.4^{a}	4.4±0.4^{b}	3.6±0.2^{b}	3.0±0.4^{a}
	15	10.6±0.3^{b}	10.7±0.6^{b}	10.1±0.2^{a}	3.9±0.2^{b}	3.6±0.3^{b}	2.8±0.7^{a}
	30	11.4±0.2^{ab}	11.8±0.2^{a}	10.1±0.7^{a}	3.2±0.2^{a}	3.0±0.2^{a}	2.7±0.4^{a}
	60	12.0±0.5^{a}	-	-	3.2±0.3^{a}	-	-
	90	12.0±0.4^{a}	-	-	3.3±0.2^{a}	-	-

Values with the same letter in each column showed no statistically significant difference $p > 0.05$; $n = 3$; analysis of variance, Duncan's *post-hoc* test); M, maceration; HAE, heat-assisted extraction; UAE, ultrasound-assisted extraction; SD, standard deviation; IC_{50}, the concentration of extract required to neutralize 50% of DPPH radicals.

The obtained results are in accordance with previously examined and published content of total polyphenols in wild thyme extracts [13], and with literature data, where the optimal particle size for the maximum antioxidant capacity of *Quercus robur* extracts was 0.5 mm [14].

The results also showed that the increase in solvent-to-solid ratio from 10:1 to 30:1 resulted in the extracts with higher scavenging potential regardless of the type of extraction method (**Table 1**). It is believed that with larger quantity of herbal material the extraction medium becomes more viscous, which results in slow release and diffusion of antioxidant compounds. On the other hand, the increase in solvent-to-solid ratio resulted in the prevention of solvent saturation, thus the increase of the content of antioxidants.

Ethanol (30-70%) extracts were more potent than aqueous extracts; 50% ethanol appeared to be the best solvent for maceration process (**Table 1**). There was no statistically significant difference between the antioxidant activities of three different ethanol extracts obtained by heat-assisted extraction, while either 50% or 70% ethanol solution can be acclaimed as the best for ultrasound-induced extraction. Miron *et al*. [15] also showed that the antioxidant activity of ethanol extracts of various aromatic plants was higher in comparison to aqueous extracts, however according to these authors there was no significant difference between 25, 50, and 75% ethanol extracts.

Duration of the extraction process seems to have the most significant effect on maceration, far less on heat-assisted and none on ultrasound-assisted extraction, regarding scavenging activity of the extracts (**Table 1**). According to the results of antioxidant potential, 30 min can be recommended for maceration and heat-assisted extraction, and 5 min for processing by ultrasonic probe, since ultrasound waves may induce generation of free radicals, polymerization and enzymatic degradation of antioxidant compounds [16,17].

The effect of liquid wild thyme extracts obtained by different extraction techniques (under the best conditions) on the neutralization of free ABTS and DPPH radicals is shown in **Figure 1**. In ABTS test, there were no differences between extracts obtained by maceration, heat- and ultrasound-assisted extraction (10.9 ± 0.5, 10.9 ± 0.3, and 10.0 ± 0.7 mmol Trolox/mL, respectively). On the other hand, in neutralization of DPPH radicals, the extracts obtained by maceration and high temperature have shown significantly lower levels of antioxidant activity, i.e. higher concentration required for scavenging of radicals (IC_{50} of 3.6 ± 0.4 and 3.3 ± 0.2 mg/mL, respectively), whereas the extracts obtained by using ultrasonic probe had statistically significantly higher antioxidant capacity (IC_{50} of 2.8 ± 0.1 mg/mL).

Numerous studies have shown that there was a positive linear correlation between polyphenol content and antioxidant potential [8,15,16]. Furthermore,

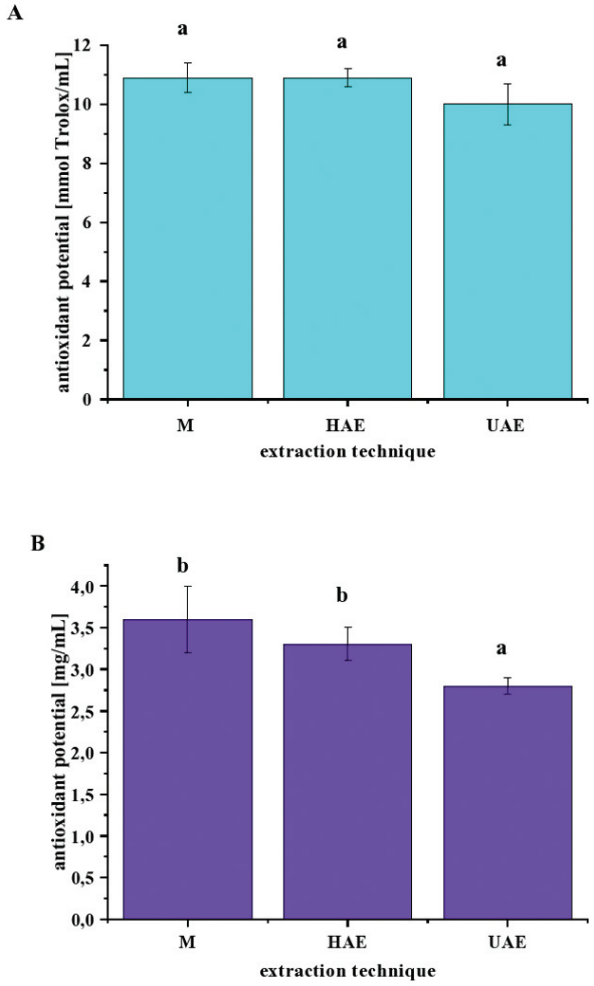

Figure 1. Mean plots of the antioxidant capacity of liquid wild thyme extracts: (A) ABTS and (B) DPPH assays; different letters (a-b) indicated that there was a statistically significant difference ($p < 0.05$; $n = 3$; analysis of variance, Duncan's *post-hoc* test); M, maceration; HAE, heat-assisted extraction; UAE, ultrasound-assisted extraction.

the antioxidant capacity of polyphenolic compounds is closely related to their structure [18]. Namely, glucosylation of flavonoids reduces their antioxidant capacity, compared to free aglycone. However, it is known that the antioxidant efficiency of flavonoids increases with the increase of number of hydroxyl groups substituted in ring B, where the *o*-dihydroxy structure allows higher stability of the formed radical and participates in electron delocalization [8,18]. The substitution of a hydroxyl group with another electron-donor group, such as an alkyl or methoxy group, increases the antioxidant potential of phenolic acids. Furthermore, there are plants whose antioxidant activity is attributed to unknown compounds or the synergistic action of multiple components as well [8].

CONCLUSION

In the present study, the effect of various particle sizes of plant material, solvent-to-solid ratios, solvent types, extraction times, and extraction procedures on the scavenging potential of liquid *T. serpyllum* extracts was examined. Presented data indicated that higher degree of herbal drug disintegration, higher solvent-to-solid ratio and the presence of ethanol within extraction medium resulted in better antioxidant activity, whereas extraction time had the most significant effect on maceration, far less on heat-assisted and none on ultrasound-assisted extraction. There were no differences between antioxidant potential against ABTS radicals of the extracts obtained by different extraction procedures. However, in DPPH assay, the extracts obtained by ultrasonic probe had statistically significantly higher scavenging capacity. Due to antioxidant properties shown in the present study, wild thyme ethanol extract may be used as an additive or adjuvant in the formulations in food, pharmaceutical, chemical, and cosmetic industries.

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CONFLICT OF INTEREST

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

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Kapacitet ekstrakta *Thymus serpyllum* L. za uklanjanje slobodnih radikala u zavisnosti od primenjenih uslova i tehnika ekstrakcije

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Kratak sadržaj

Polifenoli, kao biljni sekundarni metaboliti, poseduju snažan antioksidativni kapacitet za neutralizaciju slobodnih radikala, heliranje metala i interakciju sa lipidnim membranama, proteinima i nukleinskim kiselinama. Cilj prikazane studije je ispitivanje primenjenih uslova ekstrakcije (veličine čestica biljnog materijala, odnos rastvarač:biljna droga, vrsta rastvarača i vreme ekstrakcije) i metode ekstrakcije (maceracija, ekstrakcija na povišenoj temperaturi i ekstrakcija ultrazvučnim talasima) na sposobnost neutralizacije slobodnih radikala od strane polifenolnih ekstrakata biljke Thymus serpyllum, ispitanu primenom ABTS i DPPH metoda. Prema dobijenim rezultatima, manje čestice biljnog materijala daju ekstrakte sa boljim antioksidativnim potencijalom. Međutim, ovaj efekat je statistički značajan za ekstrakte dobijene na povišenoj temperaturi i ultrazvučnim talasima prema ABTS testu, dok je u DPPH testu efekat bio značajan samo za esktrakte dobijene maceracijom i na povišenoj temperaturi. Povećanje odnosa rastvarač:biljna droga od 10:1 do 30:1 dovodi do povećanja antioksidativne aktivnosti ekstrakata bez obzira na primenjenu tehniku ekstrakcije, dok etanolni ekstrakti pokazuju bolji potencijal u neutralizaciji slobodnih radikala u odnosu na vodene ekstrakte. Pri ispitivanju vremena ekstrakcije koje je neophodno da se dostigne najveći antioksidativni kapacitet ekstrakta, pokazano je da je 30 min optimalno vreme za maceraciju i ekstrakciju na povišenoj temperaturi, dok je za ekstrakciju ultrazvučnom sondom potrebno 5 min. Pri neutralizaciji ABTS radikala nije bilo statistički značajne razlike između T. serpyllum ekstrakata dobijenim različitim tehnikama, dok su u DPPH testu ekstrakti dobijeni primenom ultrazvučne sonde pokazali statistički značajno veću aktivnost. Zahvaljujući prikazanim svojstvima u neutralizaciji slobodnih radikala, ekstrakti biljke T. serpyllum mogu se koristiti u formulacijama u prehrambenoj, farmaceutskoj, kozmetičkoj i hemijskoj industriji.

Ključne reči: *ekstrakcija; polifenoli; kapacitet neutralizacije; Thymus serpyllum.*