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DETERMINATION OF POLYPHENOL AND FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY OF ETHANOLIC, CHLOROFORM AND ETHYL ACETATE EXTRACT OF THE PLANT SPECIES *Thymus serpyllum* L.

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ABSTRACT

This research aimed to examine the phytochemical composition, content of polyphenols and flavonoids, and antioxidant capacity of ethanolic, ethyl acetate, and chloroform extract of the plant species *Thymus serpyllum* L. The extracts were tested for the presence of alkaloids, tannins, saponins, phenolic compounds, flavonoids, steroids, terpenoids, cardiotonic glycosides, and coumarins. The total content of polyphenols was determined spectrophotometrically with the Folin-Ciocalteu reagent, according to Singleton's method, while the total content of flavonoids was determined using the method with aluminum chloride. The highest contents of polyphenols and flavonoids were determined in ethanolic extracts, where the measured values ranged from 111.00 ± 0.26 to 288.00 ± 0.23 mg GAE/g of dry extract for polyphenols, and 65.80 ± 0.19 to 198.22 ± 0.34 mg RE/g dry extract for flavonoids. The antioxidant activity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The tested extracts show a good effect on DPPH radical inhibition, with the IC50 values ranging from 35.15 ± 0.33 to 398.27 ± 0.24 µg/ml, and the greatest ability of ethanolic extract to neutralize the DPPH radical. Qualitative phytochemical analysis of the extracts showed a wide spectrum of phytochemicals, where ethanol, as the most polar solvent, extracts almost all tested phytochemicals.

Keywords: *Thymus serpyllum*, Extracts, Polyphenols, Flavonoids, Phytochemicals, Antioxidant activity.

INTRODUCTION

Healing with herbs is as old as humanity itself. The positive impact of medicinal plants on human health is explained by the presence of secondary metabolites, for example, polyphenols, which can exhibit various biological activities, such as antimicrobial, antioxidant, and anti-inflammatory. Many compounds extracted from plants are used in medicine or represent a model for the production of numerous synthetic drugs with improved pharmacological effects (Živanović, 2015).

Among the aromatic plants belonging to the Lamiaceae family, the genus *Thymus* is noteworthy for its numerous species and varieties of wild plants. Thyme species are perennial, aromatic herbs and shrubs native to Europe, North Africa, and Asia. They are commonly used as culinary herbs and flavorings. Due to their antimicrobial, spasmolytic, and antioxidant effects, they are useful for medical purposes (Stahl-Biskup & Sáez, 2002), and also shows anti-inflammatory, antinociceptive, and antitumor effects (Mahmoudi et al., 2008; Nikolić et al., 2014).

Thymus serpyllum L. (wild thyme) is a highly valued medicinal and aromatic plant whose dried aerial part is used in traditional medicine and as raw material in the pharmaceutical industry. It acts as an antispasmodic, broncholytic, expectorant, diuretic, and sedative, and has antibacterial and antimicrobial effects (Kovačević, 2003). Wild thyme contains many flavonoid and phenolic antioxidants such as zeaxanthin, lutein, naringenin, luteolin, and thymonin. Fresh thyme has a high level of antioxidants and is full of minerals and vitamins that are essential for optimal health. Its leaves are one of the richest sources of potassium, iron, calcium, manganese, magnesium, and selenium (Sharangi & Guha, 2013). Based on the above, the goal of this research was to determine the total content of polyphenols and flavonoids, evaluate the antioxidant activity and determine the presence of certain groups of phytochemicals in the ethanolic, ethyl acetate, and chloroform extracts of the plant species *Thymus serpyllum* L. collected in the area of the Šar Mountains, Serbia.

EXPERIMENTAL

Materials and methods

Chemicals and reagents

Chemicals used in the experimental work was obtained from commercial sources and was used as purchased.

UV-Vis spectroscopy

All absorbance was measured using a LLG UniSPEC 2 spectrophotometer.

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Plant material

Above-ground parts of the plant species *Thymus serpyllum* L. were collected in 2020 in the area of the Šar Mountains, Serbia. After 15 days of drying in the shade and in a drafty place, the plant material was packed in dark paper bags and stored in a dry and cool place until the analysis.

Preparation of extracts

Dried and well-ground plant material (5 g) was extracted with 100 ml of solvents of different polarity: ethanol, ethyl acetate, and chloroform, in a Soxhlet apparatus. The extraction carried out for 4 hours at the solvent boiling temperature. After the end of the extraction, the extracts were evaporated to dryness on a rotary vacuum evaporator, at a temperature of 40 ºC. The dry remains were transferred to vials and stored in a refrigerator at a temperature of 6 ºC until use. For analysis, the dry remains of the extracts were dissolved in methanol.

Determination of total polyphenol content

The total content of polyphenols in the tested extracts was determined spectrophotometrically with the Folin-Ciocalteu reagent according to Singleton's method, with minor modifications (Singleton et al., 1999). The work procedure was as follows: 0.5 ml (1000 µg/ml) of the methanol extract solution was measured in a test tube and 2 ml of Folin-Ciocalteu reagent (diluted ten times with distilled water) was added and mixed. After 3 minutes, 2.5 ml of 10% Na_2CO_3 solution was added and incubation was carried out at 25°C for 30 minutes. The absorbance was measured at 765 nm by comparing it with the blank sample (2 ml of Folin-Ciocalteu reagent was added to 0.5 ml of methanol, followed by 2.5 ml of 10% Na_2CO_3). The same procedure was repeated for the standard gallic acid solution, and the calibration curve was constructed based on different concentrations of gallic acid (7.81-500 µg/ml). Total polyphenol content was calculated from the calibration curve equation and presented in gallic acid equivalents as mg GAE/g dry extract.

Determination of total flavonoid content

The total content of flavonoids was determined spectrophotometrically, using the known method with aluminum chloride (Chang et al., 2002), with minor modifications. The working procedure was as follows: 0.5 ml (1000 µg/ml) of the methanol extract solution was measured in the test tube and 1 ml of methanol, 150 µl of 10% $AlCl_3$ solution, 150 µl of 1 M CH_3COOK solution, and 2.5 ml of distilled water were added. The contents were shaken and incubated at room temperature for 40 minutes. The absorbance was measured at 420 nm, by comparing it with the blank sample (having the same content, with replacing the standard solution of rutin and the tested extract with 0.5 ml of methanol). The same procedure was repeated for the standard rutin solutions (3.91-250 µg/ml) to construct the calibration curve. The flavonoid content was calculated from the calibration curve equation and expressed as rutin equivalent (mg RE/g of dry extract).

DPPH assay

To examine the antioxidant potential of the extracts of this plant species, we used the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, using a modified procedure according to Farasat (Farasat et al., 2014). DPPH is a stable free radical with a delocalized free electron on the nitrogen atom, so the molecule does not form the dimers with most other free radicals. This delocalization allows for the appearance of a violet color, with an absorption maximum at 517 nm. When it receives one hydrogen from an antioxidant, it is reduced and turns into yellow DPPH-H, whereby absorption intensity at 517 nm decreases. This decrease is directly proportional to the antioxidant activity of the given substance (Figure 1).

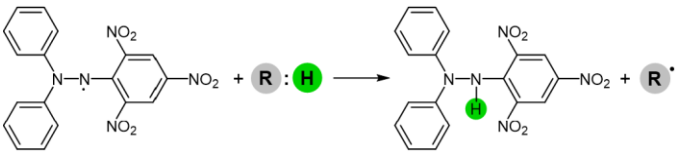

Figure 1. Mechanism of the reaction of DPPH radicals with antioxidants.

The working procedure was as follows: aliquots of 200 µl of the methanol solution of the extract of different concentrations (15.62, 32.25, 62.5, 125, 250, 500, and 1000 µg/ml) were placed in test tubes and mixed with 3 ml of a 0.004% methanol solution of DPPH. The test tubes were gently shaken and incubated for 35 minutes in a dark place at room temperature. Absorbance was measured at 517 nm. The blank sample contained methanol instead of the extract. The same procedure was repeated for the standard solution of ascorbic acid (1.96-125 µg/ml), which in our case was used as a benchmark for comparing the DPPH radical neutralization efficiency with the tested extracts, i.e., as a positive control. Inhibition of DPPH radicals in the presence of the tested sample is calculated by the formula and expressed in percent inhibition (%):

$$\% \text{ inhibition} = ((A-A_1)/A)\cdot 100$$

where A is the absorbance of the control sample (3 ml DPPH radical and 200 µl methanol), and A1 is the absorbance of the methanol extract solution (3 ml DPPH and 200 µl methanol extract solution). The IC_{50} value (µg/mL) can be calculated based on the equation of the calibration curve, which is obtained as a graphic representation of the dependence of the DPPH radical inhibition percentage on the concentration of the extract. EC_{50} "effective concentration" i.e., the IC_{50} value represents the concentration of antioxidants needed to reduce

the concentration of DPPH radicals by 50% (the lowest IC_{50} value corresponds to the highest free radical "scavenging" activity).

Statistical analysis

All analyzes were performed in triplicate and the results were statistically processed and expressed as mean value (n = 3) ± standard deviation. Statistical analyzes were performed with the help of GraphPad Prism ver. 7.00 and MS Office Excel (2016) software package.

Qualitative phytochemical analysis

During the examination of the phytochemical composition, the obtained ethanolic, ethyl acetate, and chloroform dry extracts were used as such or dissolved in a suitable solvent before certain phytochemical analyses. The extracts were tested for the presence of alkaloids, tannins, saponins, phenolic compounds, flavonoids, steroids, terpenoids, cardiotonic glycosides, and coumarins according to previously described methods (Sofowora, 1993; Trease & Evans, 2002; Harborne, 1973; Parekh & Chands, 2008; Kumar et al., 2013). These methods are based on the visual change in the color of the solution when specific reagents are added.

RESULTS AND DISCUSSION

Yield (%), total content of polyphenols and flavonoids

The yield of the obtained ethanolic, ethyl acetate, and chloroform extract of thyme ranges from 7.78 - 16.13 %. (Table 1). The highest yield during the extraction of plant material was given by ethanol as the most polar solvent (16.13%), while the yield of the chloroform extract was the lowest (7.78%).

Table 1. The yield of obtained extracts (%) of the plant species *Thymus serpyllum* L.

Extract	Ethanol	Ethyl acetate	Chloroform
Percentage yield % (w/w)	16.13	12.55	7.78

The concentration of total phenol content in the tested extracts was determined spectrophotometrically with the Folin-Ciocalteu reagent, and the values were calculated based on the equation obtained from the calibration curve. In a slightly basic medium, Folin's reagent with polyphenols gives a blue complex, whose concentration can be monitored spectrophotometrically, by measuring the absorbance on the 765 nm. The content of polyphenols in the examined extracts ranged from 111.00 ± 0.26 to 288.00 ± 0.23 mg GAE/g of dry extract (Table 2). The ethanol extract showed the highest concentration of phenolic compounds with a value of 288.00 ± 0.23 mg GAE/g dry extract, while the lowest value was measured in the chloroform extract (111.00 ± 0.26 mg GAE/g dry extract). The content of phenolic compounds in the ethyl acetate extract was 155.50 ± 0.14 mg GAE/g of dry extract. Based on numerous studies, it was seen that the total content of phenol in many plant species depends on the type of extraction, that is, on the polarity of the used solvents. Higher solubility of phenol in more polar solvents actually increases the concentration of phenolic compounds (Mohsen & Ammar, 2008; Zhou & Yu, 2004). For that reason, the total content of polyphenols in the ethanolic extract is higher, while in the chloroform extract it is the smallest. The total content of polyphenolic compounds, in our case, decreases in the expected order: ethanol > ethyl acetate > chloroform extract.

Table 2. Total content of polyphenolics and flavonoids of the plant species *Thymus serpyllum* L.

Extract	Total phenolics (mg GAE/g dw)	Total flavonoids (mg RU/g dw)
Ethanol	288.00 ± 0.23	198.22 ± 0.34
Ethyl acetate	155.50 ± 0.14	90.35 ± 0.27
Chloroform	111.00 ± 0.26	65.80 ± 0.19

The determination of the total content of flavonoids is based on their property to form complexes with metals. In this method, flavonoids form a complex with Al^{3+}, resulting in the formation of a yellow chelate. The concentration of flavonoids in the examined extracts of the plant species *Thymus serpyllum* L. ranged from 65.80 ± 0.19 to 198.22 ± 0.34 mg RE/g of dry extract (Table 2). The highest concentration of flavonoids was found in the ethanol extract (198.22 ± 0.34 mg RE/g dry extract), while the ethyl acetate and chloroform extracts showed lower concentrations (90.35 ± 0.27 and 65.80 ± 0.19 mg RE/g dry extract, respectively). As well as polyphenols, the total content of flavonoids depends on the polarity of the solvent (Min & Chun-Zhao, 2005). Therefore, the ethanolic extract has a higher content of flavonoids. In this sense, the content of flavonoids in our work decreases in the order: ethanol > ethyl acetate > chloroform extract.

The obtained results show that the examined extracts contain a smaller amount of flavonoids compared to phenolic compounds.

DPPH assay

The results showed that the tested extracts show a good effect on DPPH radical inhibition, with the IC_{50} value is in the range from 35.15 ± 0.33 to 398.27 ± 0.24 μg/ml (Table 3). The greatest ability to neutralize the DPPH radical was shown by the ethanolic extract, at a concentration of IC_{50} = 35.15 ± 0.33 μg/ml. The inhibition of the DPPH radical of the ethyl acetate extract (IC_{50} = 345.93 ± 0.10 μg/ml) is significantly lower, but still stronger in comparison to the chloroform extract (IC_{50} = 398.27 ± 0.24 μg/ml).

The IC_{50} values obtained in this way are in agreement with the results obtained for the total content of polyphenols

and flavonoids because they are known to have a strong antioxidant effect. Compared to ascorbic acid, which we know is a strong antioxidant (IC_{50} = 10.32 ± 0.16 μg/ml), we conclude that the ethanolic extract showed a high antioxidant potential.

Table 3. IC_{50} values of the tested extracts of the plant species *Thymus serpyllum* L.

Extract	DPPH assay IC_{50} (μg/ml)*
Ethanol	35.15 ± 0.33
Ethyl acetate	345.93 ± 0.10
Chloroform	398.27 ± 0.24

*Ascorbic acid was used as a positive control (IC_{50} = 10.32 ± 0.16 μg/ml)

The percentage of DPPH radical inhibition depending on the concentration of the tested extracts of the plant species *Thymus serpyllum* L. is shown in Figure 2.

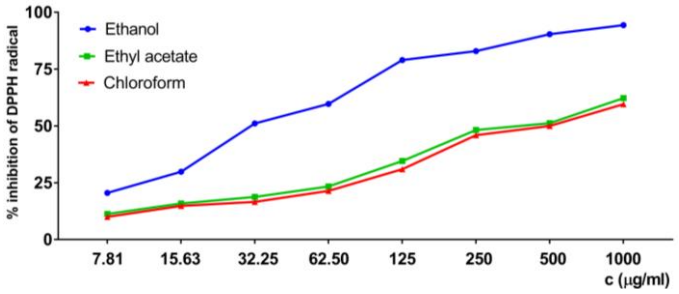

Figure 2. Percentage of DPPH radical inhibition depending on the concentration of the tested ethanolic, ethyl acetate and chloroform extract of thyme (*Thymus serpyllum* L.).

Qualitative phytochemical analysis

The examination of the qualitative phytochemical analysis showed a wide spectrum of phytochemicals in the tested extracts (Table 4). The ethanolic extract showed positive results for alkaloids, tannins, saponins, phenols, flavonoids, terpenoids, and coumarins, while the presence of steroids and cardiotonic glycosides was not confirmed. The ethyl acetate extract gave positive results for alkaloids, phenols, flavonoids, cardiotonic glycosides, and coumarins, and showed the absence of tannins, saponins, steroids, and terpenoids. The chloroform extract showed the presence of a smaller number of phytochemicals. This extract showed positive results for phenols, flavonoids, and cardiotonic glycosides, and no alkaloids, tannins, saponins, steroids, terpenoids, and coumarins were proven by the tests.

Based on the obtained results, we can conclude that the polarity of the solvent, as well as the nature of certain groups of phytochemicals, possess an important role during the extraction of plant material. Thus, ethanol, as the most polar solvent, showed the presence of almost all tested phytochemicals, while chloroform, as the least polar, showed the smallest number of phytochemicals.

Table 4. Qualitative phytochemical analysis of examined plant extracts.

Phytochemicals	Extract		
	Ethanol	Ethyl acetate	Chloroform
Alkaloids	+	+	–
Tannins	+	–	–
Saponins	+	–	–
Phenols	+	+	+
Flavonoids	+	+	+
Steroids	–	–	–
Terpenoids	+	–	–
Cardiotonic glycosides	–	+	+
Coumarins	+	+	–

CONCLUSION

Thymus serpyllum or wild thyme is a plant very useful for health and has been a synonym for folk medicine for many years. Medicinal ingredients in this plant are located only in the leaf and flower. This research showed that the extracts of this plant possess a high content of polyphenol and flavonoid compounds and significant antioxidant potential. Phytochemical analysis showed a large number of different groups of phytochemicals in all of the tested extracts. We can conclude that the presence of the phytochemicals in the extract depends on the polarity of the solvent used during the extraction. The results obtained in this work contribute to the knowledge about the antioxidant activity, and the content of polyphenolic and flavonoid compounds of the plant species *Thymus serpyllum* and, above all, will further influence the ethnopharmacological use of this plant species.

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