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Estimation of antioxidative potential of thyme *(Thymus alpestris L.)*

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Abstract
Family Lamiaceae (labia) includes a large number of cosmopolitan species including Thymus alpestris (Thyme) characterized by the presence of essential oils and phenolic compounds. It represents a perennial semi-shrubby plant. The antioxidant potential of thyme is due to the presence of polyphenolic acids and flavonoids. The topic of this paper is the extraction of thyme (Thymus alpestris) with 40% ethanol and 50% ethanol, using Soxhlet method (circulatory extraction), after which, the content of phenolics and flavonoids was determined in order to confirm the antioxidant potential of this plant species. The results show that 40% ethanol extract has the highest content of phenolics and flavonoids and the highest antioxidant potential, resultingly.

1. INTRODUCTION

Thyme (Thymus alpestris L.), from the genius Lamiaceae, is a long-lasting bushy plant. The whole plant has an aromatic scent and a pleasant taste. The Thyme ethereal oil is made by distillation of the plant itself, and is used as a fragrant and active substance in pharmaceutical and cosmetic products. It is also used as an ingredient for nutrition flavor, as well as in aromatherapy. The treatment with this ethereal oil has anti-infective, anti-viral, anti-septic, spasmolytic, and anti-bacterial effects.
The diversity and complexity of the natural combination of polyphenols in different plants makes it difficult to distinguish every single component and to estimate or compare their anti-oxidant potentials. Every plant has various phenol components and each of those components has different anti-oxidative capacity. The studies usually show a positive correlation between the content of phenol and anti-oxidative activity.
A large number of phytochemicals have anti-oxidative effects. However, polyphenol compounds attract most attention among scientists. Polyphenol compounds are secondary metabolites of plants. They have various structural characteristics, with phenol core as their basic constituent. Phenol acid and flavonoids are the most investigated materials of all polyphenols. The flavonoid group includes the following compounds: flavones, isoflavones, flavonones, flavonoids, flavanols, catechins, anthocyanidins, leucoanthocyanidins, chalcones, dihydrochalcones and aurons.
The content of etheral oil T. alpestris L. varies depending on its origin and the way in which it is produced. The percentage can vary between 0.1% and 0.6% or 0.1%and 1%. The chemical composition and yield of the Thyme ethereal oil, is also affected by its geographical origin, the phase of development, picking time, environment, and climate. Chemical polymorphism is characteristic of herbs which belong to the genius Thymus and the following species can be distinguished: geraniol, germacrene -D, citral, linalool, (E) -caryophyllene, alpha terpinyl acetate, carvacrol, thymol and many other chemotypes.
This paper investigates the anti-oxidative potential of the ethanol extractions of different concentrations after the extraction of Thymus alpestris L., (the content of phenol and flavonoids).

2. EXPERIMENTAL PART

The sample used for the purpose of the research was the dry flower of Thyme plant picked on mountain Jahorina in August 2014.
The herbal material prepared was extracted using two solvents, 40% and 60% ethanol. 5 grams of herbal material were measured, and then transferred to extraction thimble and placed to the Soxhlet extractor. A total of 100 ml of the solvent was added to the extractor, 60 ml into the distillation flask, and 40 ml into the thimble. Extraction was carried out with both concentrations of ethanol. The obtained extract was filtered using the vacuum pump, and steamed afterwards.

2.1. Determination of the content of total phenols (Follin – Ciocalte method)

The content of overall phenols in the extract of Thyme was determined using the Follin – Ciocalte method. It was expressed as the mg of the chlorogenic acid equivalent (CAE) per gram of the dry extract. The starting solution of chlorogenic acid (0.1 mg/cm^3), produced a series of

solutions with concentrations ranging from 0.001to 0.006 mg/cm^3. Next, 1.5 ml solution of Na_2CO_3 and 0.5 ml Follin-Ciocalte reagent were added into the solutions. After 30 min of incubation, absorbances were measured on SHIMADZU-UV-1800 spectrophotometer, with wavelength of 750 nm. The standard calibration curve was made representing chlorogenic acid, from where the concentrations of total phenols could be measured.

2.2 Determination of the content of total phenols (Markam method)

The content of overall flavonoids in Thyme ethanol extract was determined by the colorimetrical Markam method (1989). The dry ingredient of the Thyme was dissolved in the distilled water of different concentrations. The reaction mixture was made by mixing 1 cm^3 sample with 0.5 cm^3 $NaNO_2$. The mixture was left to incubate at room temperature for 5 minutes. After the incubation, 2.5 cm^3 $AlCl_3$ + CH_3COONa and 0.5 cm^3 NaOH (1 mol/L) were added into the mixture. The obtained mixture was filled with distilled water to the total volume of 10 cm^3. Absorbance of the sample was measured at the wavelength of 430 nm. The total content of flavonoids in the analyzed samples was expressed as the mg of the gallic acid equivalent (GAE) per gram of the dry extract.
The standard solution of gallic acid was prepared in the following manner: 50 mg of gallic acid was dissolved in 10 cm^3 of methanol, transferred to a normal 100 cm^3 cup and filled with distilled water until reaching the line on the measuring cup. In order to achieve the concentration of 0.05 mg/cm^3, 1 cm^3 of the previously made solution was transferred to a normal 100 cm^3 cup and filled with deionized water until reaching the line on the measuring cup.
A series of solutions of 0.001, 0.002, 0.004, 0.006 and 0.008 mg/cm^3 were made. Afterwards 2.5 cm^3 $AlCl_3$ + CH_3COONa and 0.5 cm_3 $NaNO_2$ were added into each solution. After half an hour absorbance was measured at 430 nm. Based on the measured absorbances, we were able to read the concentrations of total flavonoids from the calibration curve for the standard solution of gallic acid.

3. RESULTS AND DISCUSSION

3.1. Determination of total phenol content

The total content of phenols in plant extracts of Thymus alpestris L. from the area of Jahorina, obtained after extraction in the Soxhlet extractor, using 40% and 50% ethanol, was determined by the Follin-Ciocalte method. A spectrophotometrically observed change in color intensity is proportional to the concentration of phenolic compounds at a wavelength of 750 nm. The amount of total phenols in the examined samples was calculated based on the calibration curve of chlorogenic acid standard (Figure 1).

The results are expressed in mg/cm^3 (Table 1) and then converted to mg/g of dry extract in accordance with the dilution and extraction (Table 3).

Ethanol proved to be the most successful solvent in the extraction of phenolic components of many plant species [9], yielding extracts with high phenol content and high antioxidant potential. So far, it has been shown that the greatest success of phenol extraction is achieved with 40% ethanol [10,11,12], which is also experimentally proven in this paper.

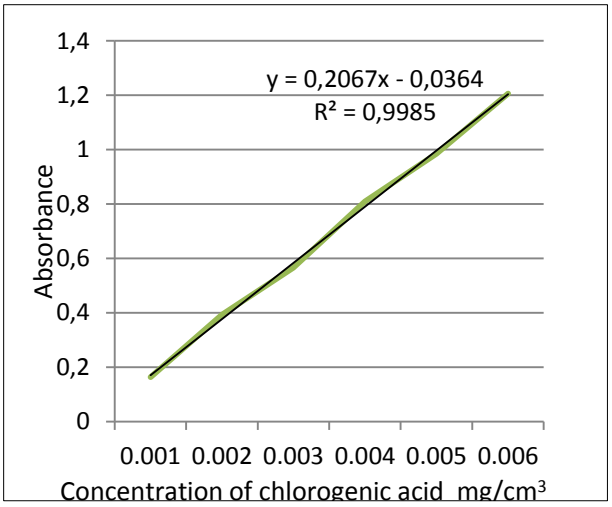

Figure 1. Calibration curve of the standard solution of chlorogenic acid for determining the content of total phenols

Table 1. Concentrations of chlorogenic acid in the standard solution, their absorbances and absorbances of the samples

Sample labels	Concentrations of HK* [mg/cm^3]	Absorbance
Standard 1	0.001	0.1638
Standard 2	0.002	0.3925
Standard 3	0.003	0.5655
Standard 4	0.004	0.8090
Standard 5	0.005	0.9857
Standard 6	0.006	1.2064
EtOH 40%	0.001599	0.3139
EtOH 50%	0.001438	0.2823

**HK-chlorogenic acid*

3.2. Determination of the total content of flavonoids

The total content of flavonoids in plant extracts (Thymus alpestris L.) obtained after extraction in the Soxhlet extractor, using 40% and 50% ethanol, was determined by the Markam method. Spectrophotometrically, a change in color intensity was observed in proportion to the flavonoid concentration at a wavelength of 430 nm. The total amount of flavonoids in the samples was calculated based on the calibration curve of the gallic acid standard (Figure 2).

The results obtained by the spectrophotometric analysis for flavonoid concentration are shown in Table 2. As in the previous analysis, it has been confirmed that using a lower concentration of ethanol in flavonoid extraction, produces a higher concentration of extracted flavonoids.

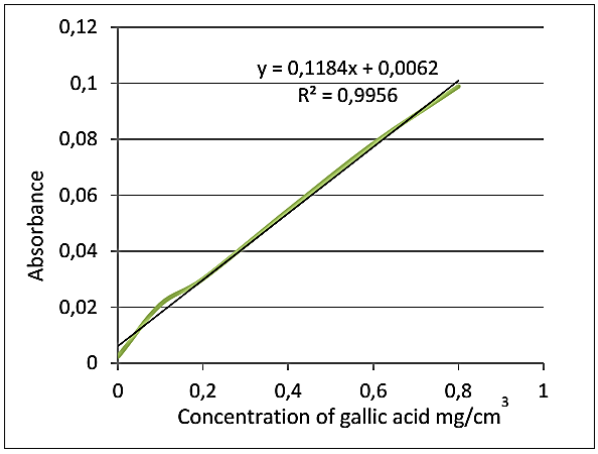

Figure 2. Calibration curve of the standard solution of gallic acid for determining the content of total flavonoids

Table 2. Gallic acid concentrations in the standard solution, their absorbances and absorbances of the samples

Sample labels	Concentrations of GAE* [mg/cm^3]	Absorbance
Standard 1	0.100	0.0211
Standard 2	0.200	0.0302
Standard 3	0.400	0.0547
Standard 4	0.600	0.0785
Standard 5	0.800	0.0990
Et-OH 40%	0.7895	0.0977
Et-OH 50%	0.7868	0.0976

**GAE-gallic acid*

3.3. The content of total phenols and flavonoids in analyzed thyme extracts (Thymus alpestris L.)

Determination of total phenol content in analyzed thyme extracts (Thymus alpestris L.) proved that 40% ethanol extract is the best extraction medium (0.03198 mg HK/g of dry extract), while using higher ethanol concentration (50%), the content of total phenol is lower and its value is 0.02876 mg HK/g of dry extract.
The content of total flavonoids also showed higher values in the ethanolic extract of lower concentration (15.79 mg GAE/g of dry extract) compared to the other ethanol extract used (15.77 mg GAE/g of dry extract).

Table 3. Total content of phenol and flavonoids in the analyzed thyme extracts (Thymus alpestris L.)

Extracts	Total phenols mg HK*/g of dry extract	Total flavonoids mg GAE**/g dry extract
40% ethanol	0.032	15.790
50% ethanol	0.029	15.770

**HK-chlorogenic acid*
***GAE-gallic acid*

The determined content of phenol and flavonoids in the extracts of thyme is shown in Diagram 1. The diagram shows that the content of total flavonoids, which are carriers of antioxidant activity, is significantly higher than the content of total phenols which, in addition to antioxidant, also show antibacterial and antiviral activity.

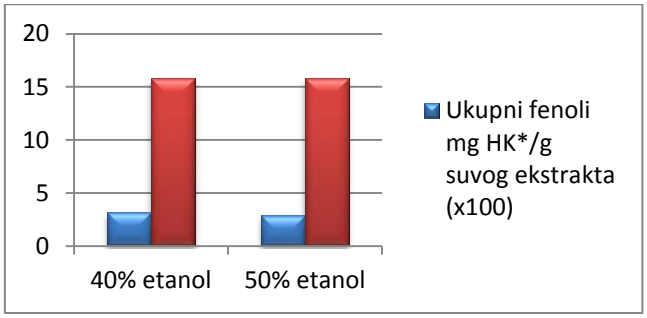

Diagram 1. The ratio of phenols and flavonoids in the examined thyme extracts (Thymus alpestris L.) to various ethanolic extracts

The obtained results of phenols and flavonoid contents indicate that thyme (Thymus alpestris L.) has a high antioxidant potential. In many previous studies it has been shown that the high content of phenolic components and flavonoids is associated with antioxidant potential. Nickavar and Esbati (2012) examined the antioxidant potential and phenolic composition of three species of the genus Thymus and in addition to high content of phenols and flavonoids, found a significant correlation between the content of flavonoids and the ability to capture free radicals [13].

4. CONCLUSION

A large number of phytochemicals have an antioxidant effect, but polyphenols have attracted the greatest attention of the researchers. The content of ethanol in the extraction solution affects the total phenol content. The total content of flavonoids shows a lower dependence on the concentration of ethanol in the extraction solution. The total content of phenol and flavonoids in thyme extracts (Thymus alpestris L.) was determined by Follin-Ciocalte and Markam method. 40% ethanol yields the highest content of total phenols while their content decreases with increasing ethanol concentration. Unlike phenols, the change in ethanol concentration does not show a greater dependence on the content of total flavonoids. The ratio of phenol and flavonoids in the examined extracts is far greater in favor of flavonoids in all the extracts used. The obtained results show that 40% ethanol extract has the highest content of phenol and flavonoids, and therefore the largest antioxidant potential. Since the antioxidative potential of thyme is confirmed, it is necessary to carry out a gas chromatographic analysis to determine the composition of the phenolic and flavonoid compounds, as well as the DPPH method for determining the antioxidant activity of the extract of this plant and the possibility of its use in medicine and food industries.

5. REFERENCES

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