Phytochemical analysis and antioxidant activity of some thymus species from Romania

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ABSTRACT

Objectives. Thymus species, T. vulgaris, T. serpyllum, T. comosus and T. glabrescens, are medicinal plants from the spontaneous Romanian flora used for their antibacterial, antispasmodic, expectorant, anti-inflammatory, anthelmintic properties, especially the plant products obtained from T. vulgaris. The aim of this study was to comparatively investigate the polyphenolic composition, as well as the in vitro antioxidant activity of some ethanolic extracts obtained from the aerial parts of thymus sp.

Materials and methods. The polyphenols, flavonoids, phenolic acids and tannins contents in the four samples thymus sp. ethanolic extracts were spectrophotometrically determined. The identification of the most important polyphenolic compounds was performed by the method of thin layer chromatography method (TLC). The antioxidant activity of hydroalcoholic extracts was evaluated in vitro by the DPPH method.

Outcomes. Following the analyzes performed, it can be revealed that there are both qualitative and quantitative differences in the polyphenolic composition of the four species of thyme studied, the richest in active principles being T. vulgaris and T. serpyllum.

Conclusions. In this paper it has been shown that thyme species are medicinal plants rich in antioxidant polyphenolic active principles, and their use for the treatment of certain diseases can be justified. In addition to the officinal species: T. vulgaris, T. serpyllum, other thyme species from the spontaneous flora of our country can be used in phytotherapy, such as: T. comosus, T. glabrescens.

Keywords: thymus species, polyphenols, antioxidant potential

INTRODUCTION

Thymus species (Lamiaceae family) are aromatic medicinal plants widespread in the Romanian flora and are rich sources of bioactive principles [1-4]. Thyme is used both in gastronomy and in the pharmaceutical industry in the production of medicines. Natural products contain volatile oil (with thymol, carvacrol), flavonoids, phenolic acids, pentacyclic triterpenoids with several therapeutic properties: antispasmodic, carminative, antibacterial, expectorant, antiseptic, antiviral, antioxidant, anti-inflammatory, anthelmintic, etc. [2-13]. The purpose
of this research was the chemical and biological analysis of some vegetal raw materials from the four species of thyme (*Thymus* species: *T. vulgaris*, *T. serpyllum*, *T. comosus* and *T. glabrescens*) in the Romanian spontaneous flora, compared to some commercial thyme teas available on the Romanian market, in order to evaluate the quality of commercial products.

**MATERIALS AND METHODS**

**Plant material**

The aerial parts of thymus sp. were collected during the flowering period (June, 2014) from the spontaneous flora of Cluj, Sibiu, Brasov: C1, C2, Cb2, Cb5, TC, TG. Five natural products purchased from Romanian commercial companies, in the form of medicinal tea, of which two were samples of *T. vulgaris* tea: C3, C4, and three were samples of *T. serpyllum*: Cb1, Cb3, Cb4 (Table 1).

| Nr. | Thymus sp.         | Origin                          | Abbreviation |
|-----|--------------------|---------------------------------|--------------|
| 1.  | *T. vulgaris*      | Spontaneous flora, Sibiu        | C1           |
| 2.  |                    | Spontaneous flora, Brasov       | C2           |
| 3.  |                    | Comercial company 1             | C3           |
| 4.  |                    | Comercial company 2             | C4           |
| 5.  | *T. serpyllum*     | Comercial company 1             | Cb1          |
| 6.  |                    | Spontaneous flora, Cindrel       | Cb2          |
| 7.  |                    | Comercial company 2             | Cb3          |
| 8.  |                    | Comercial company 3             | Cb4          |
| 9.  |                    | Spontaneous flora, Sibiu (Gura)| Cb5          |
|     |                    | Râului)                         |              |
| 10. | *T. comosus*       | Spontaneous flora, Cluj         | TC           |
| 11. | *T. glabrescens*   | Spontaneus flora, Cluj (Ciucea) | TG1          |

**Preparation of extracts**

The aerial parts of thymus sp. were dried at room temperature and then were ground to a fine powder. The plant materials (5.0 g) were extracted at 60°C using 70% ethanol, on water bath, for 30 minutes. The ethanolic extracts obtained were filtered and made up to volume (50 ml) in volumetric flasks [14-17].

**Identification of flavonoid and caffeic acid derivatives by TLC**

Thin-layer chromatography (TLC) on silica gel was used for separation and identification of the flavonoids and phenolic acids from the thymus sp. extracts. Chromatography was performed on 10 cm × 20 cm glass TLC plates coated with silica gel Si 60 F254 (Merck, Darmstadt, Germany). The ethanolic extracts were applied in 5 μL volumes as 5-mm bands, and the TLC plates were developed with the mobile phase: ethyl acetate:acetic acid: formic acid: water (100:11:11:26). Standard solutions were used: 1% methanolic solutions of: rosmarinic acid, chlorogenic acid, caffeic acid, hyperoside, rutin, quercetin and isoquercitrin. They were air dried and then sprayed with a fine spray of NEU/PEG reagents, left to dry and then visualized under UV light at 365 nm. The spots of separated compounds were marked and retention factors (Rf) were calculated and recorded [16]. The resultant chromatograms were captured on camera (Fig. 1).

**Determination of total polyphenolic content (TPC)**

Total polyphenols content was determined using the Folin-Ciocalteu technique. The vegetal extracts were diluted with 70% ethanol in volumetric flasks to 25 ml. 2 ml of these solution were mixed with 1 ml of Folin-Ciocalteu reagent and 10 ml of water and were brought into a 25 ml volumetric flask. This volumetric flask was completed with 29% sodium carbonate. After 30 minutes, the absorbance was measured at 760 nm. Gallic acid was used as standard for the preparation of a calibration curve (R² = 0.999) and the results were expressed percentage (gallic acid equivalent (GAE) g/100 g dry plant material [15,17].

**Determination of flavonoids content**

Total flavonoids content was determined using a spectrophotometric method based on flavonoid-aluminum chloride (AlCl₃) color reaction. 10 ml of the 10% plant extracts are diluted with methanol in a volumetric flask to 25 ml. The mixture obtained is left to stand for 10 minutes and after that is filtered. 5 ml
of filtered solution is taken and placed in a 25 ml volumetric flask. 5 ml of 100 g/l sodium acetate and 3 ml aluminum chloride (25 g/l) are added. The flask is completed with methanol. The absorbance of the solution was measured at 430 nm. Rutin was used as a standard for the preparation of a calibration curve (R2 = 0.999). Data were expressed as g of rutin equivalents (RE)/100 g dry plant material [14-18].

Determination of caffeic acid derivatives content

The caffeic acid derivatives content was determined using a spectrophotometric method based on phenylpropane - Arnow reagent color reaction. The plant material was extracted by refluxing with 50 ml ethanol. After this, 5 ml of the filtrate solution was diluted with 50 ml ethanol 70° in a volumetric flask (solution A). To 1 ml of solution A was added 1 ml sodium hydroxide (1N), 1 ml Arnow reagent and 10 ml water. Absorbance was measured at 500 nm with the values of phenolic acids content, expressed as caffeic acid equivalent (g CAE/100 g plant material), using an equation derived from the from the calibration curve of caffeic acid (R2 = 0.994) [14-18].

Determination of tannin content

To determine the tannins by spectrophotometry, the reactive Folin-Ciocalteu colorimetric method was used. First, the total polyphenolic compounds were determined using the Folin-Ciocalteu method and then, the same extracts were treated with 10 mg of skin powder, and the insoluble tannin-protein complexes were removed by filtration. The clear filtrate (solution of non-adsorbed polyphenols) was treated with Folin-Ciocalteu reagent as mentioned above. The tannin content was measured as a difference between the total phenolic and non-tannin content, and pyrogalol was used as the standard solution. The concentration of the tannins was calculated using the relation: C(%) = [(A control - A extract)/(A control)] x 100 where: A control = Absorbance of DPPH radical and methanol (solution containing all reagents except sample to be analyzed); A extract = Absorbance of the mixture of DPPH radical and sample extract [14-17].

RESULTS AND DISCUSSION

Thin-layer chromatography

Preliminary TLC separation and identification of polyphenolic compounds in the thymus ethanolic extracts were performed using the chromatographic system with silica gel as stationary phase and the corresponding mobile phase: ethyl acetate:acetic acid:formic acid: water (100:11:11:26). Spots of standards were easy to detect and compare with samples spots. RF values were: rosmarinic acid - 0.95 (blue spot), hyperoside - 0.62 (yellow-orange spot), rutin - 0.45 (yellow-orange spot), chlorogenic acid - 0.64 (blue spot), caffeic acid - 0.96 (blue spot), quercetrin - 0.87 (yellow-orange spot), and isoquercitrin - 0.75 (yellow-orange spot) for corresponding mobile phase mentioned earlier. The results are presented in Table 2 and Figure 1.

Rosmarinic acid was identified in almost all extracts, except T. comosus extract (TC) in which its presence was not observed. Hyperoside has been identified only in the extracts of T. comosus (TC) and T. glabrescens (TG1). Rutin has been observed especially in T. vulgaris (C1, C2, C3, C4) and T. serpyllum (Cb1, Cb2, Cb3, Cb4) but also in T. comosus was noticed its presence. Chlorogenic acid was identified in all T. vulgaris extracts (C1, C2, C3, C4) but also in some T. serpyllum samples (Cb1, Cb2). Caffeic acid has been observed in T. comosus sample (TC) and in T. glabrescens sample (TG1). Quercetrin was identified only in few samples: T. serpyllum samples (Cb3, Cb4).
Isoquercitrin was identified in all *T. vulgaris* samples (C1, C2, C3, C4) and *T. serpyllum* samples (Cb1, Cb2, Cb3, Cb4, Cb5).

**Total polyphenolic content**

The total polyphenolic content (TPC) was spectrophotometrically analyzed by a Folin-Ciocalteu colorimetric method, and the results are presented in Figure 2.

The most rich samples in total polyphenols were the extracts: Cb4 (*T. serpyllum* from commercial tea - 7.43%), Cb2 (*T. serpyllum* from spontaneous flora from Cindrel Mountains - 6.83%) and TG4 (*T. glabrescens* - 6.37%). The smallest amount of TPC was found in C4 (*T. vulgaris* commercial tea - 2.74%). *T. glabrescens* had the highest amount of total polyphenols (5.25%), followed by *T. serpyllum* (5.03%). The *T. comosus* extracts had the lowest total polyphenolic content (3.00%).

**Total flavonoidic content**

Total flavonoid content of the thymus ethanolic extracts was determined using the aluminum chloride colorimetric method, and the results were presented in Figure 3.

The highest amount of flavonoids was found in the *T. comosus* extract (1.65%). In the other samples, much smaller amounts of flavonoids were determined (0.13-0.76%), below 1%. *T. vulgaris* and *T. serpyllum* are medicinal plants with average amounts of flavonoids, *T. serpyllum* having a slightly larger amount than the garden thyme.
**Total caffeic acid derivatives content**

The contents of caffeic acid derivatives determined by spectrophotometry are shown in Figure 4.

The highest amount of phenylpropane compounds was found in *T. serpyllum* harvested from the spontaneous flora of Gura-Raului, Sibiu (Cb5 - 4.48%). Also, higher amounts of caffeic acid derivatives were obtained in another *T. serpyllum* extract, namely Cb4 (3.86%). The lowest amount of phenylpropane compounds was determined in the extract of *T. comosus* (TC - 0.05%). Large amounts of caffeic acid derivatives have also been found in the *T. vulgaris* extract (2.23%).

**Total tannins content**

The tannins content in the thymus species extracts were determined by spectrophotometry, using the Folin-Ciocalteu reagent, and the results are shown in Figure 5.

The tannins contents were determined in all thymus sp. extracts. High amounts of tannins were found in the *T. serpyllum* samples (Cb2 – 1.53%; Cb4 – 1.40%) and the *T. glabrescens* sample (TG4 - 1.47%). The smallest amount of tannins was identified in the *T. vulgaris* extract C4 (0.27%). Analyzing all species in terms of tannins content, the following results were obtained: *T. serpyllum* was the richest in tannins, followed by *T. glabrescens* and *T. vulgaris*. 
Antioxidant activity

Evaluation of the antioxidant activity of *thymus* sp. extracts was performed in vitro by the DPPH method.

An % greater than 50% shows an important antioxidant effect for which the extracts can be used in phytotherapy. The following extracts showed important antioxidant action: Cb5 (80.03% – *T. serpyllum* harvested from the spontaneous flora of Gura Riului), Cb4 (71.00% – *T. serpyllum* from a commercial tea). The other extracts showed a weaker antioxidant action. The *T. comosus* extract had lowest % (2.81%), so without antioxidant action.

CONCLUSIONS

The genus *Thymus*, used since antiquity is very well represented in the spontaneous flora of Romania, such as: *T. vulgaris, T. serpyllum, T. glabrescens, T. comosus* etc.

In this paper we have comparatively studied the polyphenolic composition of four species of thymus from the spontaneous flora of our country (*thymus* species: *T. vulgaris, T. serpyllum, T. glabrescens* and *T. comosus*), as well as from thyme species from commercial teas.

We spectrophotometrically determined the contents of total polyphenols, flavonoids, tannins and caffeic acid derivatives, as well as the *in vitro* antioxidant activity. The results of the determinations showed that thymus species are medicinal plants rich in antioxidant polyphenolic compounds, especially those from spontaneous flora, and may be important ingredients for pharmaceutical preparations.
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