BIOLOGICA NYSSANA
3 (1) • September 2012: 11-15

Original Article

Preliminary investigation of free radical scavenging activity and total phenolic content of three cultivated *Thymus* chemotypes from Bulgaria

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***Abstract*:**
Nikolova, M., Jekova, G., Dzhurmanski, A.: Preliminary investigation of free radical scavenging activity and total phenolic content of three cultivated Thymus chemotypes from Bulgaria , Biologica Nyssana, 3 (1), September 2012: 11-15.

Methanol extracts of three varieties of *Thymus* "Slava", "German winter" and "Pagane", representatives respectively of citral, thymol and geraniol chemotype were evaluated for their antioxidant potential and phenolic content. Total content of phenols was determined by spectrophotometric method using Folin-Ciocalteu reagent. The antioxidant potential of methanol extracts was estimated by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) free radical assay. All of the examined extracts exhibited significant free radical scavenging activity and their IC_{50} values were below 50 μg/mL. The highest antioxidant activity was determined for the extract of thymol chemotype "German winter" with IC_{50} value – 19.34 μg/mL. The extract of this variety had the highest amount of phenols 75.61 mg/g extract. The other two varieties "Slava" and "Pagane" have a lower content of phenols and antiradical activity. Influence of three stages of flower development on antiradical activity and total phenolic content was examined for variety "Pagane". The highest antioxidant activity was determined on the extract prepared from plant material collected at the stage "end of flowering". For the first time, provides data on the phenolic content and antiradical activity of varieties "Slava" and "Pagane". These results are the basis for selection of varieties with high antioxidant potential for the preparation of cosmetic and herbal products.

Key words: *Thymus vulgaris*, *Thymus marschalianus*, DPPH, citral, thymol, geraniol

Introduction

The genus *Thymus* L. (Lamiaceae) comprises about 300 species of perennial aromatic, herbaceous plants with many subspecies, varieties, subvarieties and forms. The plants are extensively used in phytotherapy, cosmetic and food industries (Stanev, 1974; Nikolov, 2006). Essential oils, flavonoids (highly methylated flavonoids, luteolin derivatives), biphenils and phenolic compounds have been determined as main constituents of the thyme with antioxidant, antiseptic, antiinflamatory and antimicrobial properties (Jovanović *et al*., 1995; Haraguchi *et al*., 1996; Fecka & Turek, 2008; Borosa *et al*., 2010).

The thyme shows a great species diversity which reflects to the composition of the essential oil content. Based on the dominant monoterpene in the essential oils of different *Thymus* chemotypes, compounds such as geraniol, thymol, *α*-terpineol, linalool, carvacrol can be distinguished (Thompson *et al*., 2003; Chizzola *et al*.,

2008). In recent years, several reports have been published concerning the antioxidant activity on the essential oils on different thyme chemotype (Juki *et al*., 2005; Bounatirou *et al*., 2007; Miguel *et al*., 2007; Stoilova *et al*., 2008; Grigore *et al*., 2010; Tepe *et al*., 2011) while the studies on antioxidant activity of methanol extracts from different chemotypes and varieties are insufficient (Jovanović *et al*., 1995; Kulišić *et al*., 2006). A positive correlation between antioxidant activity and phenolic content in plant extracts has been well documented (Miliauskasa *et al*., 2004; Kiselova *et al*., 2006). Widely used methods for assessing the total content of phenols and antioxidant properties of plant extracts are based on Folin-Ciocalteu reagent and 1-diphenyl-2-picrylhydrazyl (DPPH) radicals (Kiselova *et al*., 2006, Giorgi *et al*., 2009; Nićiforović *et al*., 2010; Marinova & Batchvarov, 2011).

The purpose of the present work is to examine antioxidant activity and total phenolic content varieties created and cultivated in Bulgaria: thyme "Slava" (*Thymus vulgaris*, citral chemotype) and "Pagane" (*Thymus marschalianus* Wild., geraniol chemotype) as well as of the introduced variety "German winter" (*Thymus vulgaris*, thymol chemotype). This study contributes to the knowledge of the antioxidant properties of the *Thymus* chemotypes.

Materials and methods

Plant material

The plants used for the present study were cultivated in the experimental field of Institute of Roses, Essential and Medicinal Cultures, Kazanlak. Aerial parts of two varieties "Slava" and "German winter" were collected at full flowering stage. Aerial parts of the variety "Pagane", collected in three stages of flowering – beginning of flowering, full flowering and end of flowering were analyzed.

Preparations of extracts

Air-dried, powdered plant material (1 g) was extracted with 80% methanol in an ultrasonic bath. After evaporation of the solvent the crude extract was subjected to subsequent analyses.

Determination of total phenolic content

Total phenolic content of the methanol extracts was determined by employing the methods given in the literature including Folin–Ciocalteu reagent and gallic acid as standard (Giorgi *et al*., 2009; Nićiforović *et al*., 2010). Plant extracts were diluted to the concentration of 1 mg/mL, and aliquots of 0.25 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of Na_2CO_3 (6%). After 1 h of staying at the room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract) by the following formula:

$$C = c \times V / m$$

where C - total content of phenolic compounds, mg/g plant extract, in GAE; c - the concentration of gallic acid established from the calibration curve, mg/mL; V - the volume of extract, mL; m - the weight of pure plant methanolic extract, g.

Free radical scavenging activity

Free radical scavenging activity of plant extracts was evaluated using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay **(**Choi *et al*., 2002; Stanojević *et al*., 2009**)**. Different concentrations of plant extract (10, 20, 50, 100 and 200 μg/mL, in methanol) were added at an equal volume (2.5 mL) to methanol solution of DPPH (0.3 mM, 1 mL). After 30 min at room temperature, the Ab (absorbance) values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH antiradical scavenging capacity (\%)} = [\,1 - (\,Ab_{sample} - Ab_{blank}\,) / Ab_{control}\,] \times 100$$

Plant extract solution (2.5 mL) in methanol (1.0 mL) was used as a blank, while DPPH solution in methanol was used as a control. The IC_{50} values were calculated by sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity (Software Prizm 3.00). IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH radical.

Statistical analysis

Statistical analysis was carried out using excel. All experiments were performed in triplicate. Results are presented as a value ± standard deviation (SD). Significant levels were defined at $p<0.05$.

Results and discussion

Methanol extracts of three chemotypes of Thymus (citral, thymol and geraniol) were examined for their antiradical potential using a DPPH assay and expressed as IC_{50} value- extract concentration providing 50% inhibition of the DPPH solution (Table 1).

The all examined extracts exhibited considerable free radical scavenging activity and their IC_{50} values were below 50 μg/mL. The extract of variety "German winter" (thymol chemotype) showed the strongest antioxidant activity with IC_{50} value 19.34 μg/mL, followed by citral chemotype "Slava" (33.18μg/mL) and geraniol chemotype "Pagane" (47.72 μg/mL). The established differences in the antioxidant potential of studied varieties can be explained by different chemotype – different composition of essential oils. The thyme with the highest content of thymol ("German winter") has the strongest antioxidant activity. This is in accordance with results reported by Chizzola *et al*. (2008) which prove that the essential oil with high content of thymol and/or carvacrol have the highest antioxidant activity. These data allow us to assume antioxidant potential of thyme based on the essential oil composition and individual components (geraniol, linalool, thymol, etc.).

A total phenolic content of studied varieties of Thymus "Slava", "German winter" and "Pagane" was determined (Table 1). The extracts of variety "German winter" contain the highest amount of phenols (75.61 mg/g), followed by "Slava" (56.6 mg/g) and "Pagane" (45.42 mg/g). The greater amount of phenolic compounds positively correlated to more potent radical scavenging effect.

Carried out correlation analyses shows strong positive mutual dependence between total phenolic content and antioxidant (DPPH scavenging) activity of investigated extracts (R=0, 9812). This is in accordance of several reports for such positive correlation between phenols and antioxidant activity of plant extracts (Miliauskasa *et al*., 2004; Cai *et al*., 2004; Shan *et al*., 2005; Kiselova *et al*., 2006; Villaño *et al*., 2007).

The antioxidant potential and total phenolic content of extracts obtained from material collected at differing stages of flower development of the variety "Pagane" was examined. It was found that extracts of plant material gathered at stage "end of flowering" had the highest antioxidant activity with IC_{50} value of 34.28 μg/mL (Table 2). The extracts prepared from plant material collected at the stages "beginning of flowering" and "full flowering" showed IC_{50} values 47.72 and 67.00 μg/mL, respectively. Thyme is recommended to be picked up at the "end of flowering" stage, when their antioxidant activity is 40% higher than in a "full flowering" stage and 95% higher than in stage "beginning of flowering". Opposite to the results on antioxidant activity, it was found that there are no statistical significant differences (p>0,05) in the phenolic content of the examined extracts during flowering period. Total phenols ranges from 44.08 to 46.82 mg/g. This fact suggests that radical scavenging activity of the studied extracts is not only due to phenol compounds.

Table 1. Total phenolic content and free radical scavenging activity of methanol extracts of three *Thymus* varieties

***Thymus* varieties**	**Total phenols mg GAE/g extract***	**Free radical scavenging activity IC_{50} (μg/mL)***
Pagane	46.82±1.22	47.72±5.41
Slava	56.6±1.25	33.18±1.89
German winter	75.61±2.71	19.34±1.76

* values represent mean ±SD, n=3

Table 2. Total phenolic and antiradical activity of extracts of variety "Pagane" collected at three stage of flowering

Stage of flowering	**Total phenols mg GAE/g extract***	**Free radical scavenging activity IC_{50} (μg/mL)***
beginning of flowering	44.08±1,80	67.00±7.63
full flowering	46.82±1.22	47.72±5.41
end of flowering	45.42±2.62	34.28±1.98

* values represent mean ±SD, n=3

Conclusion

In the present study, the total phenolic content and antioxidant capacity of three varieties of Thymus "Slava", "German winter" and "Pagane" were determined. High positive correlation was found between investigated indexes. Bulgarian varieties thyme – "Slava" and "Pagane" was studied for the first time for their total phenolic content and the antioxidant activity. The methanol extracts of all examined samples showed high radical scavenging activity (IC_{50} <50 μg/ml). Extracts of thymol chemotype "German winter" exhibited the strongest antiradical activity and highest content of phenols among studied varieties. Therefore, thymol chemotype can be priority for utilization in phytoterapy and cosmetics. The recommended concentration of thyme solution for lipid stabilizing in cosmetics and food products is 50 μg/ml, when it provides 95% free radical inhibition whilst for the other types of thyme, it is twice higher. Thyme is recommended to be picked up at the "end of flowering" stage, because then the content of antioxidants is the highest. Obtained data will be employed as a basis for selection of varieties with high antioxidant potential for the preparation of cosmetic and herbal products, as well as for the optimization of harvesting time.

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