Scientific Papers. Series A. Agronomy, Vol. LXIV, No. 1, 2021
ISSN 2285-5785; ISSN CD-ROM 2285-5793; ISSN Online 2285-5807; ISSN-L 2285-5785

SIMILARITY ANALYSIS OF THE POLYPHENOLIC PROFILE TO SPONTANEOUS SPECIES OF THE *THYMUS* GENUS FROM BANAT (ROMANIA)

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Abstract

This study analyses the amount of total polyphenolic content (TPC) in dry plant materials collected from different populations of Thymus belonging to several spontaneous species from multiple regions of the Banat area. The aim of the research is to identify the quantitative differences of TPC in the thyme populations studied, regarding both the area and the species, or potentially subspecies. The TPC was determined with Folin-Ciocâlteu reagent using gallic acid as standard, and the concentration of ethanolic extracts was expressed in mg/g dry matter. The results obtained were statistically analysed using proximity matrices that indicate the similarity of the polyphenolic profile in the populations studied. The populations were classified clustered according to the similarity of the polyphenolic profile. The data obtained indicate significant differences in the polyphenolic profile at species and harvest area level. The possibility of using this indicator as a chemical fingerprint for the spontaneous thyme species from Banat was analysed.

Key words*: Thymus, polyphenolic profile, wild populations.*

INTRODUCTION

Plants are an inexhaustible source of unique chemical compounds. In the last years there been was a growing need for plants used as a potential resource of new products with biological and medicinal properties. The species of the genus *Thymus* are recognized for the large amount of biologically active compounds. (Tohidi et al., 2019)

According to the latest data, about 6% of the superior plants were studied for their pharmacological potential and only 15% were evaluated for their phytochemical potential. (Cragg & Newman, 2013; Espinosa-Leal et al., 2018).

Biologically active chemical compounds are secondary metabolites in plants, chemical compounds produced by plants but which are not a condition for their growth and development. (Pickens et al., 2011) Secondary metabolites play an important role both ecologically and biologically in plant defense mechanisms often having oxidative and antimicrobial properties (Najafian, 2014; Rus et al., 2016). For humans, these compounds are of great interest, being used for various purposes since the 19th century. (Patwardhan, 2005; Cragg & Newman, 2013).

Natural antioxidants such as phenolic compounds can have significant antioxidant, anti-inflammatory and anti-proliferative properties; thus, plants can be used to prevent diseases, both as a spice and as medicinal preparations (infusions, decoctions, essential oils) (Leal et al., 2017; Rus et al., 2016; Ghitea et al., 2020).

The genus *Thymus* is taxonomically classified in the Lamiaceae Family (Labiatae), a group of medicinal and aromatic plants with a high level of polyphenolic compounds (Jovanović et al., 2017) this parameter being a characteristic of the chemical profile of species of this type (Raudone et al., 2017). It is proven that there is a direct connection between the content of polyphenols in plants and antioxidant activity with their curative effects. Polyphenols play an important role in protecting the body from the harmful effects of free radicals (Öztürk, 2015), as well as in reducing the viability of some cell lines involved in the emergence of three cancers (Martins-Gomes et al., 2018).

MATERIALS AND METHODS

The analyzed material, i.e. the above-ground vegetative part (herba), was harvested in 2018 and 2019 from several areas of Banat, located at different altitude (80-1430 m). 20 populations were analyzed, with individuals randomly selected within each population, at least 20 individuals (Table 1).

Plants were morphologically analyzed in the laboratory (phenotypic aspects). The determination was carried out with the help of specialized works (Guşuleac, 1961 in Flora României vol. VIII, Sârbu et al., 2013).

Specimen samples for each population were stored after identification in the herbarium of the Biology Department of the Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timişoara.

Table 1. Populations of the genus *Thymus* analyzed and their location

Thymus species	Sample name	Location	Altitude (m)	GPS coordinates (degree, minutes)	
Th. comosus	P1	Dobraia	890	N 44° 59'	E 22° 28'
Th. dacicus	P2	Lescovița	146	N 44° 52'	E 21° 32'
Th. dacicus	P3	Ostrov	119	N 44° 42'	E 21° 37'
Th. glabrescens	P4	Silagiu	191	N 45° 36'	E 21° 36'
Th. glabrescens	P5	Valea Iuți	119	N 44° 29'	E 22° 10'
Th. pannonicus ssp. auctus	P6	Pojejena	172	N 44° 47'	E 21° 35'
Th. pannonicus ssp. auctus	P7	Silagiu	192	N 45° 36'	E 21° 36'
Th. pannonicus ssp. auctus	P8	Tricule	82	N 44° 29'	E 22° 08'
Th. praecox ssp. janke	P9	Domogled	897	N 44° 53'	E 22° 25'
Th. praecox ssp. polytrychus	P10	Gozna	1.429	N 45° 10'	E 22° 03'
Th. praecox ssp. polytrychus	P11	Semenic	1.399	N 45° 11'	E 22° 04'
Th. praecox ssp. polytrychus	P12	Semenic	1.395	N 45° 11'	E 22° 04'
Th. pulegioides ssp. chamaedrys	P13	Carasova	593	N 45° 09'	E 21° 52'
Th. pulegioides ssp. chamaedrys	P14	Dobraia	924	N 44° 59'	E 22° 28'
Th. pulegioides ssp. chamaedrys	P15	Pojejena	194	N 44° 47'	E 21° 35'
Th. pulegioides ssp. montanus	P16	Carasova	511	N 45° 09'	E 21° 52'
Th. pulegioides ssp. montanus	P17	Pojejena	170	N 44° 47'	E 21° 35'
Th. pulegioides ssp. pulegioides	P18	Carasova	582	N 45° 09'	E 21° 52'
Th. pulegioides ssp. pulegioides	P19	Semenic	998	N 45° 13'	E 22° 04'
Th. pulegioides ssp. pulegioides	P20	Nermet	389	N 45° 14'	E 21° 53'

Sample preparation and determination of total phenolics content (TPC)

The extraction method used was conducted according to the Cocan et al., 2018 method with modification regarding extraction time and conditions. Sample of 0.5 g of herb was weighed accurately on analytical balance. 20 mL ethylic alcohol 70% (1:10, w/v) were added to the flask and vortexed for 2 min (Vortex Genie 2, Scientific Industries Portland, Oregon United States). The extracts obtained were filtered and stored at room temperature (24-25°C) until used. TPC was determined with Folin-Ciocâlteu reagent using gallic acid as standard (Dumbrava et al., 2020). Briefly, 0.5 mL of ethanolic extract was mixed with 1.25 mL of Folin-Ciocâlteu reagent (Merck, Germania) 1:10 diluted with distilled water. This mixture stands for 5 min at room temperature. Therefore, was added 1.0 mL of Na_2CO_3 (60g/L). That mixture stands for 30 min at 50°C in darkness. The absorbance at 750 nm was measured using a UV-VIS Analytic Jena Specord 205 Spectrophotometer in triplicate. TPC was calculated based on the standard curve of gallic acid, and results were expressed as milligrams of gallic acid equivalent per gram of extract (mgGAE/g extract).

Statistical methods used

Statistical analysis of experimental data has been carried out with IBM SPSS Statistics Version 21. Cluster analysis of polyphenolic profiles can be used to determine significant differences between the chemotypes of the different *Thymus* populations analyzed. *Thymus* populations were analyzed statistically and clustered according to the similarity of the polyphenolic profile, using the function

"Hierarchic cluster Analysis". The method used is "between-groups linkage" and the distance is the Euclidean one. On the basis of these distance coefficients, UPGMA dendrograms were generated for cluster analysis of polyphenolic profiles in the populations studied. Average values, standard deviations, maximum and minimum content of all results obtained were determined.

RESULTS AND DISCUSSIONS

The evaluation of biochemical diversity between some *Thymus* populations based on the total polyphenol content of the alcoholic extract of dry plant material revealed at the 20 populations studied, an average of 0.779 ± 0.006 mgGAE/g dry vegetable mass (herba).

The median of TPC values determined in the studied populations is of 0.580 ± 0.334 mgGAE/g dry vegetable mass (herba). The amount of TPC in the *Thymus* populations studied, can be seen in Figure 1.

The total values of the polyphenol determined by our study in *Thymus* species were found to be lower than those reported in the literature for plants collected from natural habitats or grown under field conditions (Petrović et al. 2017; Tohidi et al., 2019; Guriță et al., 2019; Golkar et al., 2020).

Climatic and pedological conditions, sampling and extraction methods, the genetic background of the plants is responsible for the differences in the TPC content reported in various studies.

In our conditions, the extraction time (2 min.) and the extraction method (vortexing) were adapted to the purpose of the study, namely to compare the TPC content of different species prelevated from spontaneous flora and not to quantify the TPC content. These changes in the extraction technique were one of the factors responsible for the lower amounts of TPC reported in existing literature studies.

A maximum of 2.253 ± 0.020 mgGAE/g dry vegetable mass (herba) was determined at the population level, at population 6, species *Th. pannonicus* ssp. *actus* – identified in the area of Pojejena and a minimum quantity of 0.204 ± 0.004 mgGAE/g dry vegetable mass (herba) in population 2, *Thymus dacicus* – identified in the area of Lescovița.

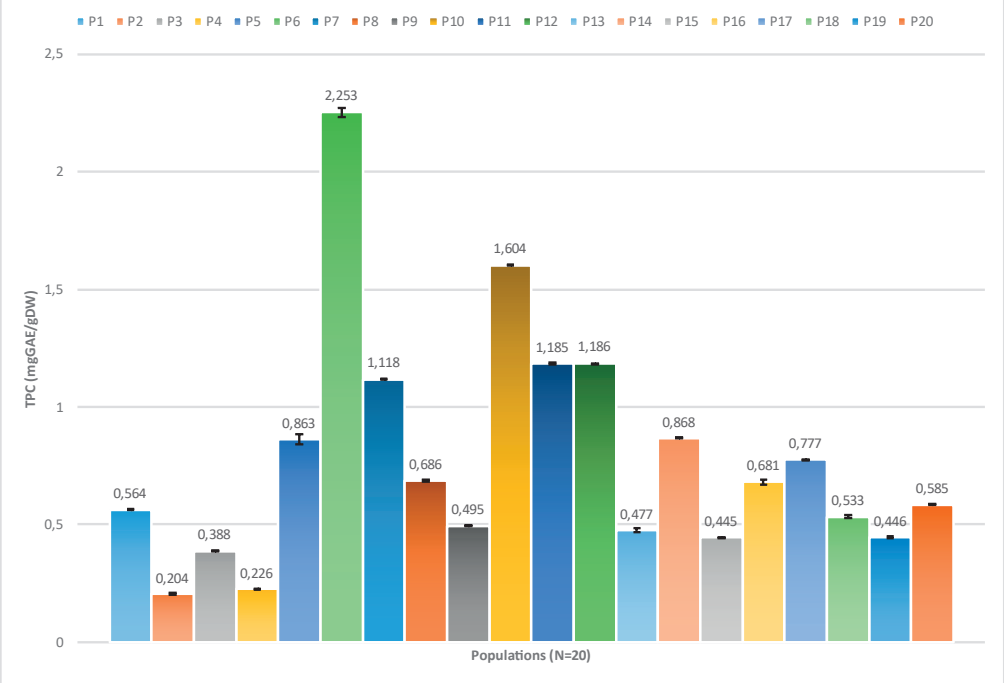

Figure 1. The amount of TPC in *Thymus* populations studied (mgGAE/gDW)

At the species level, within the six species evaluated in our study, the largest differences were obtained in the species *Th. pannonicus* ssp. *actus* (three populations), where the average amount of TPC determined was 1.352 ±0.809 mgGAE/g dry vegetable mass (herba). The largest amount of TPC 2.253 ± 0.020 mgGAE/g dry vegetable mass (herba) was determined at the population 6, identified in the area of Pojejena locality and the lowest amount of TPC 0.686 ± 0.003 mgGAE/g dry vegetable mass (herba), was determined in population 8 identified in the area of Tricule.

The genus *Thymus* presents a high ecological plasticity, occupying various areas, which explains the high chemical variability (Varga et al., 2015; Raudome et al., 2017).

An interesting situation occurs at populations 9, 10, 11 and 12 respectively, belonging to the same species, *Th. praecox,* where the average quantity of TPC determined was 1.117 ± 0.459 mgGAE/g dry vegetable mass (herba), where a difference at subspecies level occurs: a significantly higher amount of polyphenols was determined in populations 10, 12 and 11 of *Th. praecox* ssp. *polytrychus,* with an average of 1.325 ± 0.242 mgGAE/g dry vegetable mass (herba), harvested in the Gozna-Semenic area, compared to *Th. praecox* ssp. *janke*, harvested in the area of the Domogled massif, where the average amount of TPC determined was 0.495 ± 0.001 mgGAE/g dry vegetable mass (herba). Being an endemic species (Boros et al. 2010; Raudome et al., 2017), with limited spread, these differences could provide additional chemotaxonomic characteristics with possible use in subspecies taxonomic determination. There may also be a possible link between the significantly increased quantity determined in these populations and the altitude at which these species grow, but further studies are needed on this issue.

Significant variations were also identified in the two populations of *Th. glabrescens,* with an average TPC of 0.545 ± 0.450 mgGAE/g dry vegetable mass (herba), where the amount of TPC determined at population 18, harvested in the Iuti Valley was 0.863 ± 0.049 mgGAE/g dry vegetable mass (herba) is significantly higher than that determined in population 2, harvested in the area of Silagiu locality, of 0.226 ± 0.001 mgGAE/g dry vegetable mass (herba). Here we can also discuss a possible zonal influence, the distance between the harvesting areas being the largest.

For the two populations of *Th. dacicus,* the quantity of TPC determined had the lowest values, with an average quantity of 0.296 ± 0.130 mgGAE/g dry vegetable mass (herba), and a population difference of 0.184 ± 0.004 mgGAE/g dry vegetable mass (herba). Previous studies have indicated in this species a quantity of TPC of 178.83 ±1.09 mgGAE/g dry vegetable mass (Petrović et al. 2017). These values are superior to the values obtained by us, but the parameters used in the determination were different, in particular the method of extraction of polyphenols, which differ essentially (enriched and concentrated methanolic extract).

At the population of *Th. comosus*, identified in the area of Dobraia, the quantity of TPC determined was of 0.564 ± 0.002 mgGAE/g dry vegetable mass (herba). As an endemic species, this result may have significance, but further studies are needed to validate the result obtained.

The eight populations of *Th. pulegioides* were analyzed, where the average quantity of TPC determined was of 0.601 ± 0.159 mgGAE/g dry vegetable mass (herba), where a smaller differentiation in subspecies from *Th. praecox*. Thus, in the case of the three populations of *Th. pulegioides* ssp. *pulegioides*, there were recorded values below the other two subspecies, with an average of 0.521 ± 0.070 mgGAE/g dry vegetable mass (herba), followed by the three populations of *Th. pulegioides* ssp. *chamaedrys*, with an average of 0.597 ± 0.236 mgGAE/g dry vegetable mass (herba). The highest values were determined at *Th. pulegioides* ssp. *montanus* with an average of 0.729 ± 0.068 mgGAE/g dry vegetable mass (herba). At the subspecies level, the largest value differences were recorded at *Th. pulegioides* ssp. *chamaedrys* with a range of variation in the amount of TPC between 0.445 ± 0.001 and 0.868 ± 0.002 mgGAE/g dry vegetable mass (herba). In this case, a possible altitudinal influence was also observed. The smaller differences in value were recorded at *Th. pulegioides* ssp. *montanus* with a range of variation in the amount of TPC between 0.681 ± 0.010 and 0.777 ± 0.002 mgGAE/g dry vegetable mass (herba).

The results obtained give a significant variation in the content of TPC by species and harvesting area. Quantitative differences in the altitude of the harvesting area have been identified and further studies are needed, with high variability in the data obtained.

There are numerous studies showing that these quantitative variations of TPC are mainly determined by species, genotypic differences, extraction method, phenological period, sample processing conditions, climatic and environmental factors (Tohidi et al., 2017; Tohidi et al., 2019; Golkar et al., 2020).

Depending on the total amount of polyphenols determined, the matrix of dissimilarity of the populations taken in the study was drawn up, then used to obtain the dendrogram (Figure 2) according to the method of the mean of the groups (clusters). A number of two major clusters of *Thymus* populations were obtained by cluster analysis relative to the amount of TPC determined in this study in the 20 *Thymus* populations, to which was added population 6, which by the much higher amount of TPC determined was framed separately.

Two subclusters were identified at the level of the first major cluster (cluster 1). The first subcluster (1.1) unites a number of 10 populations with an average content of 0.436 ± 0.130 mgGAE/g dry vegetable mass (herba), with a minimum quantity of 0.204 ± 0. 004 mgGAE/g dry vegetable mass (herba) in second population and a maximum quantity of 0.585 ± 0.002 mgGAE/g dry vegetable mass (herba) in population 20.

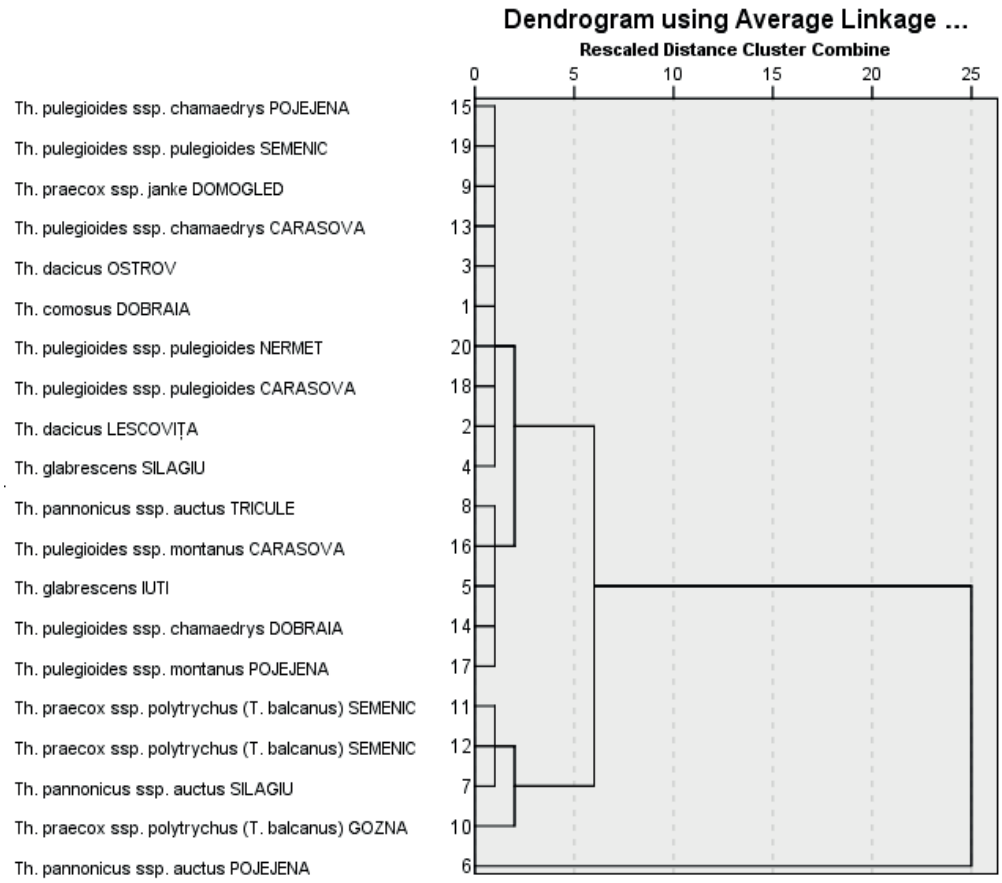

Figure 2. Two-dimensional dendrogram obtained by cluster analysis of the amount of TPC in *Thymus* populations (N=20)

The second subcluster (1.2) of the first major cluster, unites five populations, with an average content of 0.775 ± 0.091 mgGAE/g dry vegetable mass (herba), with a minimum amount of 0.681 ± 0.010 mgGAE/g dry vegetable mass (herba) at population 16 and a maximum quantity of 0.868 ± 0.002 mgGAE/g dry vegetable mass (herba) at population 14. The TPC content of populations in major cluster one is lower than populations in major cluster two.

Major Cluster two is distinguished by higher values in terms of the amount of TPC and comprises a subcluster (2.1) with three populations, with an average quantity of 1,163 ± 0,039 mgGAE/g dry vegetable mass (herba), of which a minimum amount of 1,118 ± 0,001 mgGAE/g dry vegetable mass (herba) at population seven and a maximum quantity of1,186 ± 0,001 mgGAE/g dry vegetable mass (herba) at population twelve, to which population ten is added, with a significantly higher amount of TPC 1.604 ± 0.001 mgGAE/g dry vegetable mass (herba).

Statistical data on the amount of TPC in population clusters of *Thymus* are given in Table 2.

A significant aspect of this study is the positioning in different clusters of two subspecies of the same species *Thymus praecox*, a result that may represent a valid starting point for the development of biochemical fingerprinting methods of *Thymus praecox* subspecies. Thus, Population 9 - *Th. praecox* ssp. *janke,* identified in the area of the Domogled massif was classified in cluster 1, and population 10, respectively population11 and 12 - *Th. praecox* ssp. *polytrychus (T. balcanus),* in the Gozna-Semenic area was placed in cluster 2.

Table 2. Statistical data of clusters for Thymus populations studied, based on total polyphenol content (N=20)

Cluster/ subcluster	Mean	STD[1]	Min.[2]	Max.[2]	CV[3] (%)
Subcluster 1.1 (N=10)	0.436	0.130	0.204 ± 0.004	0.585 ± 0.002	29.8
Subcluster 1.2 (N=5)	0.775	0.091	0.681 ± 0.010	0.868 ± 0.002	11.7
Subcluster 2.1 (N=3)	1.163	0.039	1.118 ± 0.001	1.186 ± 0.001	3.3
Subcluster 2.2 Popultion 10	1.604	0.001	-	-	-
Population 6	2.253	0.020	-	-	-

[1]STD = standard deviations.
[2]Min./Max. = minimum/maximum TPC content (means ± standard deviations mgGAE/g).
[3]CV=coefficient of variation.

CONCLUSIONS

The results obtained highlight a significant variation in the content of TPC in *Thymus* populations from Banat area by species and harvesting area.

The degree of variability of the values obtained in widespread species indicates that further development of genotypic studies is necessary to allow a possible correlation between the species of the genus *Thymus* at chemical and genetic level.

A validation of these studies could provide additional taxonomic characteristics with possible use in the development of faster taxonomic determination tools.

Depending on the similarity of the quantity determined by total polyphenols the populations of *Thymus* were classified hierarchically into two main clusters; positioning in different clusters of two subspecies of the same species *Thymus praecox*, a result that may represent a valid starting point for the development of biochemical fingerprinting methods of *Thymus* subspecies.

ACKNOWLEDGMENTS

The chemical research was performed in the Interdisciplinary Research Platform (IRP) belonging to Banat University of Agricultural Sciences and Veterinary Medicine King Michael I of Romania from Timişoara, Romania.

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