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CONTRIBUTIONS TO THE STUDY OF THE BIOCHEMICAL VARIABILITY OF THE POLYPHENOLS EXISTING IN 8 *THYMUS PULEGIOIDES* POPULATIONS FROM NORTHERN MOLDAVIA

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Abstract: *In the frame of some investigations performed to establish the chemical variability in the polyphenolic fraction of some plants belonging to the Lamiaceae family, we studied 8 Thymus pulegioides populations harvested in the spontaneous flora of Northern Moldavia in 2010. The polyphenolic fraction was investigated by thin layer chromatography, spectrophotometric determinations of the polyphenolic components and of the derivates of polyphenolcarboxylic acids, as well as by high performance liquid chromatography. The determinations were performed on methanolic extracts (DER 1:200 g/ml) and their concentrates (DER 0.125:1 g/ml). We could notice the constant presence of some quantitative variations between the analyzed samples, but qualitative differences could not be highlighted. If the flavonoid content varied between 0.1425 and 0.2845 g% equivalent rutoside, in case of the polyphenolcarboxylic acids the determined values were between 1.7201 and 4.0715 g% equivalent caffeic acid. To be noticed that both the maximum concentrations and the minimum regarding the content of flavonoids and that of polyphenolic acids is to be found in the same populations (Brosteni and Farcasa-1). In case of the vegetal material originated from three different populations from close areas (Farcasa 1,2,3) the quantitative differences are also significant.*

Key words: *Thymus pulegioides, polyphenolic fraction, flavonoids, polyphenolcarboxylic acids.*

1. Introduction

If at the beginning of the last century over 450 species were registered as belonging to the *Thymus* genus, nowadays it is accepted that this genus contains at most 150 species, of which 75 are found in the European flora [1].

The European *Thymus* species used in therapeutics are the following: for essential oil and herba: *Thymus capitatus* Hoff. et Link., *T. serpyllum* L., *T. vulgaris* L.;

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only for herba: *Thymus* ×*citriodorus* (Pers.) Schreb. (= *T. pulegioides* × *T. vulgaris*); and only for essential oil: *Thymus mastichina* L., *T. pulegioides* L., *T. zygis* L.

Since the existing data in the scientific literature of Romania on the polyphenolic fractions of Thymus pulegioides are scarce, as the only detailed mention found was about a cultivated population collected from the University Botanical Garden of Medicinal Plants, University of Medicine and Pharmacy of Targu-Mureş [2], we hereby aimed to study the qualitative and quantitative examination of flavonoids and phenylcarboxylic acids derivatives of 8 samples collected in June 2010 from the spontaneous flora of Northern Moldavia.

As one knows, some aspects of the chemical composition and bioaccumulation level of secundary metabolites are not only genetically determined for every species but also ecologically conditioned. A number of environmental factors such as soil composition and quality, temperature, altitude, light regime, hydric balance, and plant density per unit area are important determinants shaping the chemistry of plants. Our study aims to precisely highlight the chemical variability of the polyphenolic fraction depending on a number of factors mentioned above.

2. Material and methods

The origins of the samples collected in May-June 2010 is presented in table 1.

The origins and the labels of the "Thymus pulegioides" samples Table 1

Sample	Location	Collection date (2010)	Altitude (m), aprox.
Tp1	Albesti / Botosani county	24.05	75
Tp2	Brosteni / Suceava county	06.06	650
Tp3	Farcasa 1 / Neamt county	06.06	600
Tp4	Farcasa 2 / Neamt county	06.06	600
Tp5	Farcasa 3 / Neamt county	06.06	600
Tp6	Potoci / Neamt county	06.06	600
Tp7	Valea Putnei / Suceava county	06.06	880
Tp8	Vama / Suceava county	06.06	600

The phytochemical analysis aimed both qualitative and quantitative aspects of the polyphenolic fraction for each of the natural populations; it consisted of thin-layer chromatography separations, spectrophotometric determinations and high-performance liquid chromatography (HPLC) analysis.

Our study was performed on methanolic extracts (DER=1:200 g/mL) for spectrophotometric determinations and on their concentrates (DER=0.125:1 g/mL) for chromographic analyses.

Thin-layer chromatography was due to a previously published method [3].

The spectrophotometric determination of the flavonoids mainly aimed the capacity of these compounds to form intense yellow complexes in the presence of Al^{3+} cations, for which the absorbance is measured at λ= 430 nm; the results were expressed as rutoside equivalents in whole mass percent.

The spectrophotometric determination of the phenolcarboxylic acids was performed by treating the methanolic extracts in alkaline medium (natrium carbonate) with phosphowolframic acid, it was blue coloured, the absorbance is measured in λ = 660 nm; the results were

expressed as caffeic acid equivalents in whole mass percent [4]. For the determinations we used a ABL&E Jasco V550 UV-Vis spectrophotometer.

The determination of the phenolic acid and flavonoid composition of the *Thymus pulegioides* samples was performed by means of a reverse phase HPLC-UV method carried out using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprised of a quaternary solvent delivery system, an on-line degasser, an autosampler, a column temperature controller and UV-photodiode array detector (DAD) coupled with an analytical workstation; Agilent Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm); column temperature: 30 °C; detection wavelength: 320 nm; flow rate: 1 mL/min; gradient elution: acetonitrile (solvent A) and 2 mM aqueous sodium acetate solution adjusted to pH 3.5 with glacial acetic acid (solvent B); the initial conditions were 2% A and 98% B; the linear gradient programme was of 2-14-20-30-25% solvent A at 0-20-40-50-60 min, after which we switched back to the initial conditions; sample injection (2 µL) was performed by an autosampler programme.

As standards we used caffeic, chlorogenic, ferulic, and rosmarinic acids, the flavonoids: apigenin, apigenin 7-O-glucoside, hyperoside, luteolin, luteolin 7-O-glucoside, rutin, quercetin, (LG-Standards). To generate the calibration curve, the standard stock solutions were diluted with methanol and analyzed in the same conditions.

3. Results and discussion

By means of thin-layer chromatography separation of the extracts of *Thymus pulegioides* samples we obtained the chromatograms pictured in figures 1 and 2.

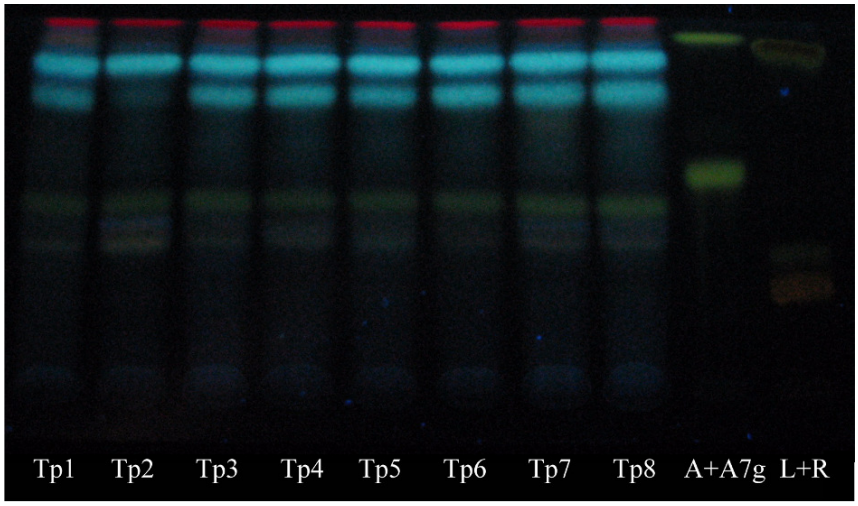

Fig. 1. *TLC chromatogram for flavonoids from concentrated methanolic extracts (DER=0.125:1 g/mL) of Thymus pulegioides (2010). Samples: Tp1: Albesti, Tp2: Brosteni, Tp3: Farcasa-1, Tp4: Farcasa-2, Tp5: Farcasa-3, Tp6: Potoci, Tp7: Valea Putnei, Tp8: Vama. Standards: A: Apigenin, A7g: Apigenin 7-glucoside, L: Luteolin, R: Rutoside.*

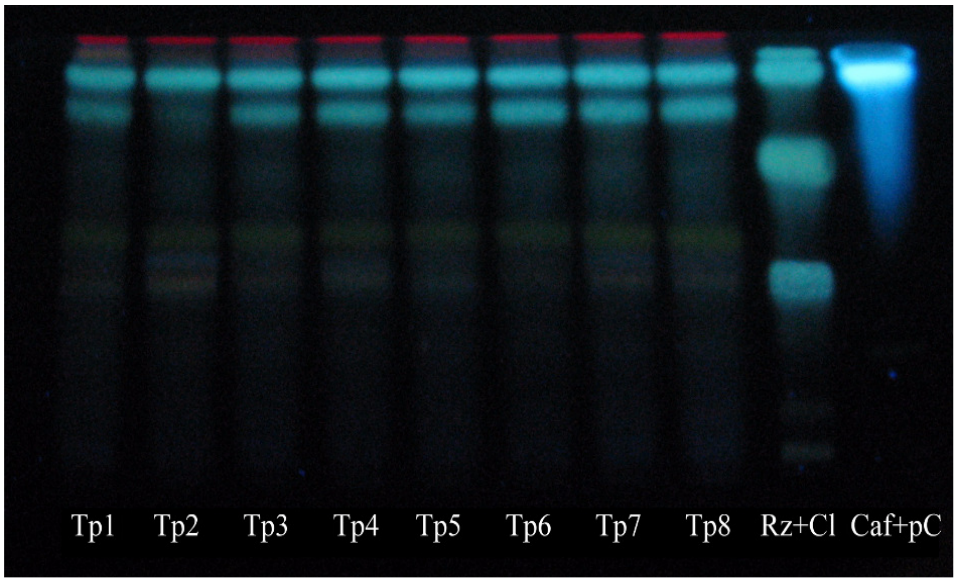

Fig. 2. *TLC chromatogram for phenolic acids from concentrated methanolic extracts (DER=0.125:1 g/mL) of Thymus pulegioides (2010). Samples: Tp1: Albesti, Tp2: Brosteni, Tp3:Farcasa-1, Tp4: Farcasa-2, Tp5: Farcasa-3, Tp6: Potoci, Tp7: Valea Putnei, Tp8: Vama. Standards: Rz: Rozmarinic acid, Cl: Chlorogenic acid, Caf: Caffeic acid, pC: p-Coumaric acid.*

As one can notice, the spectrum of polyphenolic compounds seems to be similar to all the samples, but from the variation of the intensity of the spots we conclude that there are quantitative variations between populations analyzed. Thus, regarding flavonoids and polyphenols acids it seems that the Vama population has one of the largest amounts of these substances while Farcasa-1 population has the weakest spots. The best represented is the spot for rosmarinic acid, as confirmed by HPLC analysis which revealed that rosmarinic acid has the highest amount for all samples studied (see Table 3).

If we observe the chromatograms for the three populations from Farcasa, we notice their quantitative variation, population Farcasa-2 showing the most intense spots, while the population Farcasa-1 appears to have the lowest content of polyphenolic components. Phytochemical analysis showed the existence of a low variability between geographically close plant populations (e.g. *Valea Putnei* and *Vama* populations); only in the case of *Farcasa* populations the variability is slightly pronounced. Out of the flavonoids used as standards, the samples investigated show the presence of apigenin and apigenin 7-glucoside (as also assayed by HPLC).

By complementing the qualitative determination with spectrophotometric quantification, we obtained for the 8 populations of *Thymus pulegioides* analyzed in 2010 the results summarized in Table 2.

Table 2

Content of polyphenolic compounds in Thymus pulegioides by spectrophotometric determinations

Sample	**Location**	**Total flavonoid content** [g *rutoside* equivalents / 100g dried vegetal material]	**Total polyphenol content** [g *caffeic acid* equivalents/ / 100 g d.v.m.]
Tp1	Albesti	0.2953±0.0008	2.0459±0.0109
Tp2	Brosteni	0.8245±0.0011	4.0715±0.0087
Tp3	Farcasa 1	0.1425±0.0009	1.7201±0.0064
Tp4	Farcasa 2	0.3743±0.0010	2.2181±0.0188
Tp5	Farcasa 3	0.2917±0.0014	2.2486±0.0203
Tp6	Potoci	0.2992±0.0004	2.4198±0.0119
Tp7	Valea Putnei	0.3616±0.0008	3.3437±0.0107
Tp8	Vama	0.3256±0.0007	2.4660±0.0177

By the graphical representation of the table values (Figures 3 and 4), we discover that the Brosteni sample has a maximum content of over 0.8% flavonoids expressed as rutoside equivalents and of more than 4% phenolic acids expressed as mass percent caffeic acid equivalents.

Of all the samples investigated the Farcasa-1 population turns out to be the poorest in flavonoids. Within each group, flavonoids or phenolic acids, we found a reasonably large chemical variability which is perhaps best represented by the 3 populations from Farcasa.

Table 3

The semiquantitative determination by HPLC of flavonoids and phenolcarboxylic acids in concentrated extracts (DER=0,125:1 g/mL) of "Thymus pulegioides" 2010 samples

Sample	**Location**	**mg/100g dried vegetal material**			
		Chlorogenic acid	***Rosmarinic acid***	***Apigenin 7-O-glucoside***	***Apigenin***
Tp1	Albesti	0.88	103.78	0.37	1.20
Tp2	Brosteni	0.15	146.46	0.04	0.74
Tp3	Farcasa 1	0.16	99.35	* <d.l.	0.91
Tp4	Farcasa 2	0.09	109.41	0.06	0.61
Tp5	Farcasa 3	1.35	107.07	0.06	0.74
Tp6	Potoci	0.08	102.49	0.05	0.66
Tp7	Valea Putnei	0.09	167.64	0.13	0.74
Tp8	Vama	0.45	172.32	0.11	1.21

** <l.d. : value under the detection limit*

Switching to the HPLC analysis of the samples investigated, we identified in concentrated extracts the four components listed in table 3.

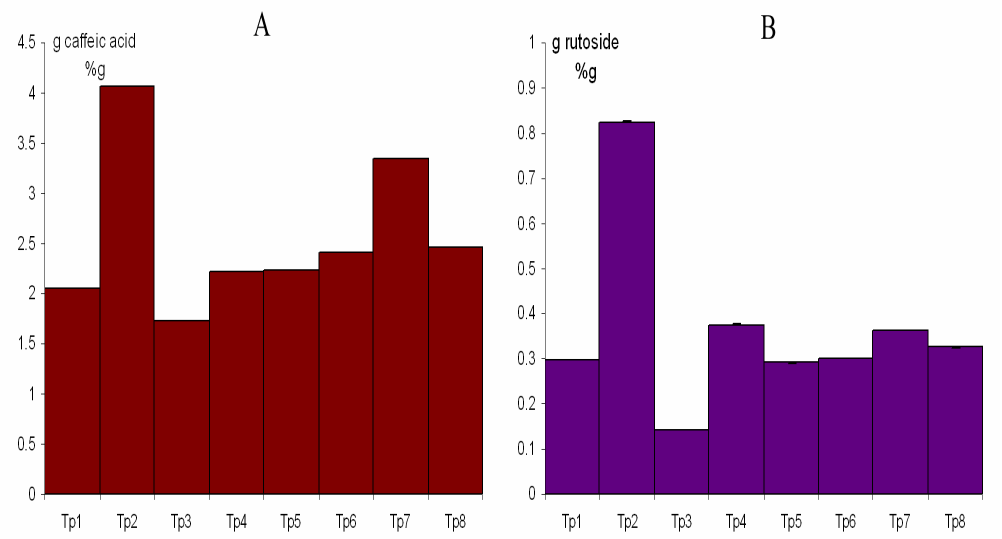

Fig.3. **A:** *Variation in total polyphenol(**A**) and flavonoid(**B**) content of Thymus pulegioides samples determined by spectrophotometric technique. Samples: Tp1=Albesti; Tp2=Brosteni; Tp3=Farcasa-1; Tp4=Farcasa-2; Tp5=Farcasa-3; Tp6=Potoci; Tp7=Valea Putnei; Tp8=Vama.*

One example of a chromatographic curve obtained by HPLC analysis of the concentrated extract of *Thymus pulegioides* sample from Brosteni population is illustrated in Figure 4.

As shown in Table 3 we identified two phenolic acids in *Thymus pulegioides* samples namely rosmarinic and chlorogenic acids, the former as a major component. Regarding the flavonoids, we succeeded to identify only two compounds, namely apigenin and apigenin 7-O-glucoside, in the absence of appropriate standards.

In order to better visualize the variability of polyphenol content of *Thymus pulegioides* samples, we plotted graphically in Figure 5 the values determined by HPLC for rosmarinic acid, which is a major compound and the differences are more obvious.

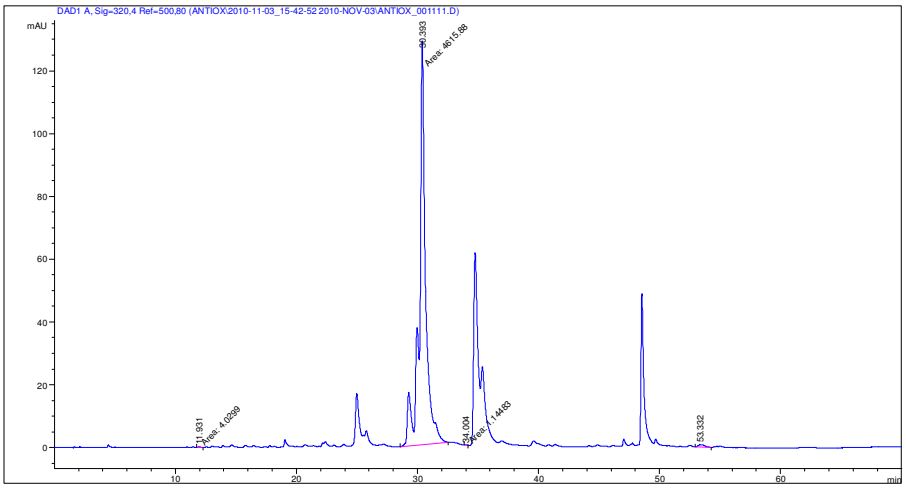

Fig. 4. *Extracted HPLC chromatogram for the concentrated extract of Thymus pulegioides sample from Brosteni population.*

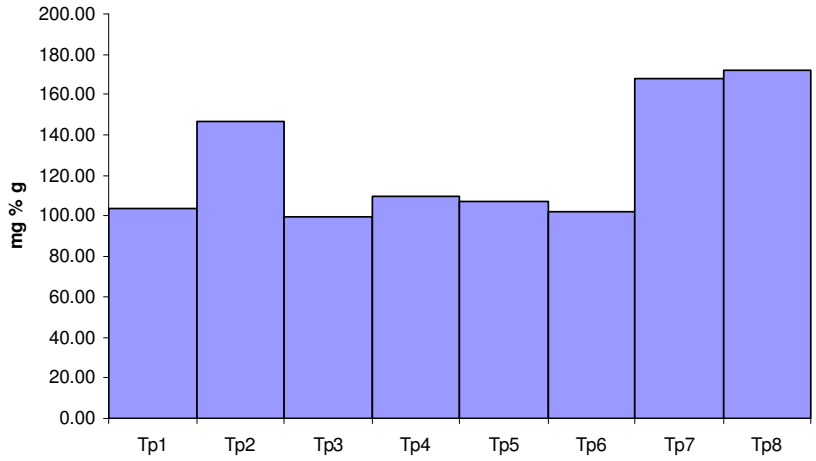

Fig. 5. *The variability in rosmarinic acid content determined by HPLC for "Thymus pulegioides" samples.*
Samples: Tp1=Albesti; Tp2=Brosteni; Tp3=Farcasa-1; Tp4=Farcasa-2; Tp5=Farcasa-3; Tp6=Potoci; Tp7=Valea Putnei; Tp8=Vama.

4. Conclusion

Analyzing the data presented we conclude that there is a fairly large chemical variability regarding the quantitative aspect of the various polyphenolic components even if they are considered to be fixed substances (and thus less dependent on soil and climate conditions). In qualitative terms we could not detect any differences – this fact could be explained by the genetic determination of the *Thymus pulegioides* species.

5. Aknowledgement

The paper was presented at the: al IV lea Simpozion National de Etnofarmacologie cu participare internationala cu tema "Etnofarmacologia la interfata bioaliment-fitomedicament" - iunie 2011 Brasov – Sirnea.

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