Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVII, Fascicula 4, 2016

CONTRIBUTIONS TO THE PHYTOCHEMICAL STUDY OF THE POLYPHENOLIC FRACTIONS SEPARATED FROM *THYMUS PULEGIOIDES* L. NATURAL POPULATIONS HARVESTED IN NORTHERN ROMANIA

RADU NECULA[1], ELVIRA GILLE[1], VALENTIN GRIGORAŞ[1], IRINA BOZ[2*], URSULA STĂNESCU[3]

Received: 03 October 2016 / Revised: 04 October 2016 / Accepted: 19 October2016 / Published: 30 November 2016

Keywords: *Thymus pulegioides* L., polyphenols, HPLC-UV
Abstract: *Thymus pulegioides* L. represents one of the plant species that are part of *Serpylli herba*, a drug used in therapeutics as a stomachic, carminative, expectorant and diuretic and also in nutrition as a flavouring agent. In our study we evaluated the quantitative determination of polyphenolcarboxylic acids and flavonoids from 24 samples of *T. pulegioides* collected from spontaneous flora in North-East Romania. We also aimed for the identification of some components by means of high-performance liquid chromatography. We detected the presence of a high chemical variability of the polyphenolic fraction going up to the level of the different bioactive components. To better control the pharmaceutical quality of the plant material it would be desirable to introduce this species into culture.

INTRODUCTION

Thymus L. is one of the largest genuses of the *Lamiaceae* family, which based on its high polymorphism and its large variety of culture sorts; it still presents some important taxonomic challenges. Some reference publications (Blashek et al., 2006; Heywood and Richardson, 1972) estimated the total number of species for this genus to be about 150 out of which 75 species belong to the European flora.

The *Thymus pulegioides* L. species belongs to the *Serpyllum* (Miller-Bentham) section, it grows spontaneously in the southern zone up to the high plateau of Abyssinia, in the North reaching Greenland and the European and Siberian Arctic, and in the East reaching the Kamchatka Peninsula and Japan.

As a pharmaceutical product, *T. pulegioides* is part of the *Serpylli herba* mixture drug, actually constituted of several species of *Thymus*, namely: *T. pannonicus* All., *T. austriacus* Bernh., *T. dacicus* Borbas, *T. marschallianus* Willd., *T. glabrescens* Willd. and *T. pulegioides* L. The product may contain up to 0.6% essential oil, its composition varying depending on the drug source. It also contains tannins of the labiates type up to 3% and flavonoids (Czygan, 1997; Wagner and Bauer, 1999). A recent paper (Shekarchi et al., 2012) published by a research group from Iran studying 29 species of lamiaceae plants out of which 4 species of *Thymus*, established one of the best methods for selective extraction of rosmarinic acid and its quantitative determination by the high-performance liquid chromatography (HPLC) technique. The authors have identified for *Thymus citriodorus* a significant high concentration of rosmarinic acid, a component with antioxidant and antimicrobial activity.

The essential oil from *Thymus pulegioides* received little attention in the past. However, in the last decade a few research groups have focused on different chemotypes that are present in spontaneous populations of wild thyme (De Martino et al., 2009; Groendahl et al., 2008; Loziene and Venskutonis, 2005; Loziene et al., 2003, Mockute and Bernotiene, 1999; 2001; 2005). Among these, there is a study that refers to the essential oil separated from *Thymus pulegioides* of Romanian origin (Pavel et al., 2010).

In 2012, a chemical and microbiological study on the volatile oil of *Thymus pulegioides* was published by Spanish authors (Pinto et al., 2006), who evidenced that the major components are carvacrol and thymol. Antifungal activity was investigated on seven clinical strains of *Candida*, five of *Aspergillus* and on five human dermatophytes. The essential oil of *Thymus pulegioides* has been proved to develop a clinically relevant inhibitory action of fungi. To investigate the mechanism of action the authors used the flow-cytometry technique to monitor the integrity of cytoplasmic membrane and the level of ergosterol for the studied fungi; the essential oil has damaged the membrane, reducing its ergosterol content.

The *Serpylli herba* product is used in traditional medicine as stomachic, carminative, expectorant, and in the treatment of bladder or kidney infections, but it is also used for food flavoring. As decoctions or tinctures, the plant product is used in phytobalneotherapy for the treatment of rheumatic pain; the tinctures can also be used in the massage therapy. In Romanian folk medicine (Butură, 1979), the mixture of wild thyme, comfrey (*Symphytum officinale*) and common hop (*Humulus lupulus*) was administered internally for common cold and rheumatism.

In this study we aimed to investigate the intraspecific and intrapopulational chemical variability of *Thymus pulegioides* L. specimens collected from northeastet of Moldavia region (Romania).

MATERIALS AND METHODS

Plant material

We collected 24 samples (June, 2012) consisting of the flowering aerial parts (*herba*) of *Thymus pulegioides*, originating from 12 villages (Vama, Valea Putnei, Lunca Putnei, Lunca Broșteni, Mădeni, Frumosu, Farcașa, Dreptu, Galu, Potoci, Doina Arini and Ortoia) from the northern Moldavia region of Romania. Depending on the size of the spreading area we collected one sample or more subpopulation samples. The plant material was collected from clearing sites close to forest edge. Voucher specimens were deposited at the herbarium of the "Stejarul" Biological Research Centre, Piatra Neamt, Romania.

Sample preparation

Powdered plant material (2.5g) was weighed accurately and extracted under reflux 3 times with methanol (30 mL), the extracts were combined into a 100-mL volumetric flask, and finally, the plant residue was washed with methanol, adjusting the volume to 100 mL. For each extract, the absorbance at 660 nm was measured 3 times spectrophotometrically, respectively, then each sample was submitted 3 times for HPLC analysis.

Instrumentation and chromatographic conditions

Thin-layer chromatography was prepared according to the method of H.Wagner and S.Bladt (1996). Spectrophotometric determinations were carried out using a Jenway 6300 VIS spectrophotometer. The spectrophotometric determination of the phenolcarboxylic acids was performed by treating the methanolic extracts in alkaline medium (natrium carbonate) with phosphowolframic acid, which coloured in blue the samples, the absorbance was measured at λ = 660 nm; the results were expressed as caffeic acid equivalents for the whole mass percent. 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 for the whole mass percent (according to Romanian Pharmacopoeia).

The cromatographic analysis were carried out using a Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprised of a quaternary solvent delivery system, an on-line degasser, a column temperature controller and UV-photodiode array detector (DAD) coupled with an analytical workstation; Agilent Zorbax Eclipse XDB-C18 reverse-phase column (4.6 × 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: 2-14%A in 20 min, 14-20%A in 20 min, 20-30%A in 10 min, 30-25%A in 10 min, after which we switched back to the initial conditions; sample injection (10 µL) was performed by an autosampler programme. Chromatographic peaks were confirmed by comparison of the values for retention time and the UV spectra of reference substances. Quantitative determination was finalized by means of external calibration method.

RESULTS AND DISCUSSIONS

The study of the polyphenolic fraction was carried out by thin layer chromatography for polyphenolic acids and flavonoids (Wagner and Bladt, 1996), spectrophotometric determination and HPLC analysis.

By thin layer chromatography we detected the presence of small quantities of flavonoid compounds, while the spots of phenolic acids were clearly outlined. The spectrophotometric determination has revealed the existence of intraspecific chemical variability for the polyphenolic fraction and also of intrapopulational variability if one compares each different subpopulations collected from Frumosu, Farcasa, Dreptu or Potoci localities.

Of the analyzed samples, the highest content in phenolic acids was detected in plant material collected from Ortoaia (sample 24), followed by a sample from Dorna-Arini (sample 23). The smallest concentrations were determined for samples from Farcasa (no. 4 and 5), for which we also had two subpopulations rich in this type of active principles.

The flavonoid content, as already observed by thin layer chromatography, was reduced, 6 of the 24 samples showing concentrations below 0.1%.

In order to determine the nature of some of the polyphenols present in the plant material we applied HPLC analysis, by which we could determine, based on available standards, the presence of rosmarinic acid, chlorogenic and caffeic acids, and also the flavonoids apigenol, apigenol-7-O-glucoside and luteolin (Table 1). The data reveals that rosmarinic acid is the component present in the highest amounts in the plant product, while apigenol-7-O-glucoside is the major flavonoid. Our determinations could not reveal the presence in the plant material of rutoside or luteolin-7-O-glucoside. In such case it should be required that the results of spectrophotometric determinations to be expressed as the found reference substances namely, rosmarinic acid and apigenol-7-O-glucoside for more accurate interpretation.

The existence of intraspecific and intrapopulational chemical variability was evidenced by means of spectrophotometry, and confirmed by HPLC analysis.

Table 1. Polyphenolic derivates identified in *Thymus pulegioides* L. samples by means of HPLC analysis

No.	**Population**	**mg/g dried plant material**					
		Chlorogenic acid	Caffeic acid	Rosmarinic acid	Apigenol -7-O-glucoside	Luteolin	Apigenol
1	Vama 1	0.55	0.07	10.36	0.07	0.05	0.08
2	Vama 2	0.20	0.06	12.46	0.07	0.06	0.12
3	Valea Putnei	0.09	0.08	20.16	0.39	0.16	0.13
4	Lunca Brosteni 1	0.09	0.07	18.08	0.34	0.13	0.19
5	Lunca Brosteni 2	0.05	0.06	13.50	0.29	0.11	0.10
6	Mădei	0.20	0.05	17.40	0.38	0.09	0.07
7	Frumosu 1	0.05	0.06	6.40	0.07	0.03	0.04
8	Frumosu 2	0.44	0.05	16.55	0.30	0.09	0.11
9	Frumosu 3	0.08	0.08	7.25	0.05	0.06	0.09
10	Fărcașa 1	0.05	0.05	8.02	0.07	0.05	0.13
11	Fărcașa 2	0.09	0.05	20.19	0.25	0.14	0.14
12	Fărcașa 3	0.14	0.04	16.34	0.22	0.07	0.09
13	Fărcașa 4	0.07	0.05	5.78	0.00	0.05	0.08
14	Fărcașa 5	0.05	0.06	4.19	0.01	0.05	0.14
15	Fărcașa 6	0.06	0.05	8.76	0.22	0.07	0.10
16	Fărcașa 7	0.10	0.06	7.40	0.06	0.09	0.14
17	Dreptu 1	0.23	0.06	15.40	0.24	0.07	0.07
18	Dreptu 2	0.10	0.06	9.76	0.18	0.05	0.07
19	Galu	0.09	0.05	6.53	0.05	0.09	0.16
20	Potoci 1	0.08	0.05	7.63	0.03	0.06	0.11
21	Potoci 2	0.15	0.05	7.85	0.04	0.06	0.05
22	Potoci 3	0.16	0.06	6.99	0.06	0.05	0.11
23	Dorna Arini	0.23	0.09	14.36	0.19	0.09	0.10
24	Ortoaia	0.15	0.08	16.93	0.42	0.14	0.11

By comparing the results of the two determinations, spectrophotometry *versus* HPLC (Table 2), we find that there is a large difference in terms of the values for the quantity assayed by spectrophotometry compared with the results obtained from HPLC analysis, which indicates that other polyphenolic derivates are present in significant amounts in the extracts.

Table 2**.** Polyphenol content determined spectrophotometrically compared with the total phenolic acids and flavonoids identified by HPLC

No.	**Population**	**Polyphenolcarboxylic acids [mg per g]**		**Flavonoids [mg per g]**	
		Spectro	**HPLC**	**Spectro**	**HPLC**
1	Vama 1	15.98	10.98	1.54	0.20
2	Vama 2	16.41	12.73	1.27	0.25
3	Valea Putnei	25.76	20.32	4.00	0.68
4	Lunca Broșteni 1	22.27	18.25	3.13	0.67
5	Lunca Broșteni 2	16.60	13.61	1.71	0.50
6	Mădei	23.82	17.65	3.39	0.54
7	Frumosu 1	13.49	6.50	0.65	0.15
8	Frumosu 2	22.50	17.04	3.27	0.50
9	Frumosu 3	15.75	7.42	1.47	0.20
10	Fărcașa 1	12.10	8.12	0.89	0.24
11	Fărcașa 2	20.40	20.33	3.81	0.52
12	Fărcașa 3	19.63	16.52	1.72	0.38
13	Fărcașa 4	9.85	5.90	0.56	0.14
14	Fărcașa 5	9.46	4.30	0.57	0.21
15	Fărcașa 6	15.28	8.88	1.61	0.39
16	Fărcașa 7	15.51	7.57	1.16	0.29
17	Dreptu 1	16.91	15.69	2.71	0.38
18	Dreptu 2	12.41	9.92	1.03	0.30
19	Galu	10.31	6.67	0.91	0.29
20	Potoci 1	13.18	7.76	0.76	0.19
21	Potoci 2	13.88	8.05	1.09	0.15
22	Potoci 3	10.47	7.20	1.03	0.21
23	Dorna Arini	24.52	14.68	3.62	0.38
24	Ortoaia	26.69	17.16	5.19	0.68
25	Pârâul Cârjei	21.26	16.87	3.23	0.41
26	Pârâul Pintei	16.06	7.21	1.24	0.25

The table shows that the samples from Galu (19) has the lowest content of phenolic acids determined by HPLC, while the plant population of Ortoaia (24) has the highest content. But if we try to ascertain the proportion by which we were able to identify by HPLC the components of phenolic acid type from the total amount determined spectrophotometrically, it presents similar percentages for the two samples: 64.66% in the first case and 64.30% in the second.

When comparing the subpopulations sampled from Vama, Lunca Brosteni, Dreptu, Potoci, Farcasa and Frumosu, the samples with the lowest values proved to be two from Farcasa (13.14), while the richest samples came from Frumosu (8) and Brosteni (4). For these samples, the identification of polyphenolics acids by HPLC as percentages from the total amount determined spectrophotometrically was approx. 60% for Farcasa and 76% for Frumosu, respectively, 82% for Lunca Brosteni.

By means of HPLC analysis we were able to quantify a low content of flavonoids, consisting of apigenol, apigenol-7-O-glucoside and luteolin. Similar to the phenolic acids determinations, the sample from Galu (19) has the lowest content in flavonoids, the total sum of the amount for the

compounds identified by HPLC accounting for only 32%. In the case of the sample collected from Ortoaia (24), it turns out to be among the richest in flavonoid content, yet the percentage of identification by HPLC is just 13%.

From the rest of the samples, the subpopulations of Frumosu and Farcasa stand out. Sample no. 7 representing a subpopulation of Frumosu has a low flavonoid content, the same as samples no. 13 and 14 collected from Farcasa. Calculating for these samples the recovery percent of the three flavonoid components identified, we found out that for the Frumosu sample the value is 23%, while for the Farcasa samples it is 24% and 36%, respectively.

The highest amount of flavonoids are assayed for one sample from Farcasa (11) and one from Frumosu (8), yet the identification proportion by HPLC, is only 14% and 15%, respectively. The results show that the samples analysed contain other flavonoid compounds in significant amounts.

Our data confirm our previous observation (Necula et al., 2011) that the biosynthesis level of polyphenolic derivatives varies within the *Thymus pulegioides* populations (probably depending on pedoclimatic conditions of the place of origin), as observed from determinations of total amounts as well as in the case of each individual component.

CONCLUSION

The phytochemical analysis carried out on polyphenolic fractions from samples of *Thymus pulegioides* L. collected from 12 localities from the north-east of Romania in the year of 2012 has demonstrated the existence, in the plant material sampled from the wild flora, of a high chemical variability concerning the level of bioactive compounds. Important components of the polyphenolic fraction are rosmarinic, caffeic and chlorogenic acids, apigenol, apigenol-7-glucoside and luteolin which also vary from a sample to another in large limits. In this situation, it would be important to cultivate the species in monitored agrotechnical conditions, to offer the patients a medicine vegetal product of a good quality.

ACKNOWLEDGMENTS

This work was supported by a grant of the Romanian Ministry of Education, CNCS–UEFISCDI, project number PN-II-RU-PD-2012-3-0307.

REFERENCES

Blashek W., Ebel S., Hackenthal E., Holzgrabe U., Keller K., Reichling J., Schultz V., (2006): *Hagershandbuch der Drogen und Arzneistoffe*, 7th Edition, Springer, Berlin, Germany

Butură V., (1979) “Enciclopedie de etnobotanică românească” (in Romanian), Editura Ştiinţifică şi Enciclopedică, Bucureşti, Romania, pp. 66-67

Czygan F.C., (1997): *Serpylli herba, in „Teedrogen und Phytopharmaka”*, edited by M. Wichtl, 3rd Edition, WFG, Stuttgart, Germany

De Martino L., Bruno M., Formisano C., De Feo V., Napolitano F., Rosselli S., Senatore F., (2009): *Chemical Composition and Antimicrobial Activity of the Essential Oils from Two Species of Thymus Growing Wild in Southern Italy*, Molecules, 14(11), 4614-4624

“Farmacopeea Română” (Romanian Pharmacopoeia), 10th Edition (in Romanian), Medical Publishing House, Bucureşti, Romania, 1993.

Groendahl E., Ehlers B.K., Keefover-Ring K., (2008): *A new cis-sabinene hydrate chemotype detected in large thyme (Thymus pulegioides L.) growing wild in Denmark*. J Essent Oil Res, 20, 40–41

Heywood V.H., Richardson I.B.K., (1972): *Labiatae, in „Flora Europeea“*, edited by T.G. Tutin, V.H. Heywood, N.A. Burges *et al.*, (Vol.3), Cambridge University Press, Cambridge, UK

Loziene K., Venskutonis P., (2005): *Influence of environmental and genetic factors on the stability of essential oil composition of Thymus pulegioides*, Biochem. Syst. Ecol., 33, 517–525

Loziene K., Vaiciuniene J., Venskutonis P.R., (2003): *Chemical composition of the essential oil of different varieties of thyme (Thymus pulegioides L.) growing wild in Lithuania*. Biochem Syst Ecol, 31,249-59

Mockute D., Bernotiene G., (1999): *The main citral-geraniol and carvacrol chemotypes of the essential oil of thymus pulegioides L. growing wild in vilnius district (Lithuania). J*. Agric. Food Chem 47(9), 3787-90

Mockute D, Bernotiene G., (2005): *Chemical composition of the essential oils and the odor of Thymus pulegioides L. growing wild in Vilnius*. J Essent Oil Res. 17, 415–418

Mockute D., Bernotiene G., (2001): *The alpha-terpenyl acetate chemotype of essential oil of Thymus pulegioides L.*, Biochem. Syst. Ecol., 29(1), 69-76

Necula R., Aprotosoaie A.C., Cioanca O., Gille E., Stanescu U., (2011): *Contributions to the study of the biochemical variability of the polyphenols existing in 8 Thymus Pulegioides populations from Northern Moldavia*; Bulletin of the Transilvania University of Brasov, Series VI: Medical Sciences; Vol. 4 (53); No. 2; 15-22.

Pavel, M., Ristic, M., Stevic, T. (2010): *Essentials oils of Thymus pulegioides and Thymus glabrescens from Romania: chemical composition and antimicrobial activity*, J. Serb. Chem. Soc. 75(1), 27-34

Pinto E., Pina-Vaz C., Salgueiro L., Gonçalves M.J., Costa-de-Oliveira S. *et al.*, (2006): *Antifungal activity of the essential oil of Thymus pulegioides on Candida, Aspergillus and dermatophyte species*. J. Med. Microbiol., 55(Pt 10),1367-73

Shekarchi M., Hajimehdipoor H., Saeidnia S., Gohari A.R., Hamedani M.P., (2012): *Comparative study of rosmarinic acid content in some plants of Labiatae family*. Phcog Mag, 8(29), 37-41

Wagner H., Bauer R., (1999): In *„Arzneidrogen und ihre Inhaltsstoffe“*, 6th Edition, WFG, Stuttgart, Germany, pp.108-110

Wagner H., Bladt S., “Plant Drug Analysis – A Thin Layer Chromatography Atlas”, 2nd Edition, Springer, Berlin, Germany, 1996.

1 - NIRDBS/”Stejarul” Biological Research Centre, Piatra-Neamț, Romania
2 - Institute of Biological Research, Iași, Romania
3 - “Gr.T.Popa” University of Medicine and Pharmacy, Department of Pharmacognosy-Phytotherapy, Iasi, Romania
* boz_irina@yahoo.com