CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS FROM FIVE POPULATIONS OF *THYMUS PULEGIOIDES* L.

IRINA BOZ, ELVIRA GILLE,[*] RADU NECULA,[*,**] SIMONA DUNCA[***] and MARIA-MAGDALENA ZAMFIRACHE[***]

Institute of Biological Research, 47, Lascăr Catargi Str., 700107, Iasi, Romania
[*]*Stejarul Biological Research Centre, 6, Alexandru cel Bun Str., 610004, Piatra Neamt, Romania*
[**]*"Gr. T. Popa" University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Pharmacognosy-Phytotherapy, 16, University Str., 700115, Iasi, Romania*
[***]*"Alexandru Ioan Cuza" University, Faculty of Biology, 20A, Carol I Blvd., 700506, Iasi, Romania*
✉ *Corresponding author: Irina Boz, irina.boz@uaic.ro*

Received November 6, 2013

Knowing that the species of the *Thymus* genus present important therapeutic properties, in this paper we aim to analyze the essential oils from five spontaneous populations of *Thymus pulegioides* L. in order to identify populations with potential therapeutic value. For this purpose, essential oils were extracted by hydro-distillation, using a NeoClevenger type apparatus according to the European Pharmacopoeia standards. The separation and the identification of the components were carried out using GC-MS (gas chromatography coupled with mass spectrometry). Antibacterial testing was performed on two test microorganisms, *Streptococcus pyogenes* ATCC 19615 and *Pseudomonas aeruginosa* ATCC 27853, using the diffusion method and the microplate method. With respect to the chemical composition of the essential oils, there were significant qualitative and quantitative differences. It was also noted that all essential oils inhibited the growth and development of the two test microorganisms used.

Keywords: *Thymus pulegioides*, essential oils, antibacterial activity

INTRODUCTION

Lemon thyme is an aromatic plant used both for medicinal purposes and as a spice almost all over the world. The genus *Thymus* L. is very frequently found in the Mediterranean region, where it does not exceed 50 cm in height, being well adapted to drought and heat.[1] In the Romanian flora, 17 *Thymus* species can be found, 16 of which are spontaneous and only one (*Thymus vulgaris* L.) is cultivated.[2] A common feature of the species belonging to this genus is the presence of the secretory hairs under different forms, hairs that contain volatile oils with inviting fragrances; this is probably one of the reasons for which man was attracted by these plants, analyzing the oils for various uses.[1]

Concerning the volatile oils belonging to the species of the *Thymus* genus, we noticed great chemical variability.[3,4,5,6,7] We found 8 chemotypes in *Thymus pulegioides* L. volatile oil.[8] The chemical variability of the volatile oils may be caused both by intrinsic factors (seasonal and ontogenetic variations), as well as extrinsic ones (medium factors as altitude, soil, climate, photoperiod etc.).[9]

At present, there are numerous studies regarding the antibacterial activity of the *Thymus* volatile oils, with the aim to identify compounds with antibacterial activity.[10,11,12,13,14] Thymol and carvacrol seem to play an important role in this aspect. These phenolic terpenes are linked to the amino- and hydroaminic groups of the proteins from the bacterial membranes, altering their permeability and leading to death of the bacterium.[15]

In the present paper, we intended to determine the chemical composition of the volatile oil from

5 *Thymus pulegioides* populations and to test their antibacterial activity, aiming to identify the spontaneous populations with therapeutic potential.

EXPERIMENTAL

Plant material

The vegetal material is represented by *Thymus pulegioides*, a species that grows in the Romanian wild flora. The aerial parts of this plant were collected in the anthesis period, from 5 different zones (Farcasa – 3 different zones, Vama and Secuieni).

The plant has an underground thick, wooden rhizome, which forms highly branched, vigorous stems that are flexuous, upward and arched at the basis. The floriferous branches are seriated being covered with reverse, small hairs on the edges. The leaves are ovate to sub-round and elliptical, glabrous, attenuated into an unciliated petiole. The bracteas are similar to the leaves. The inflorescence is spiciform elongated, interrupted and capitates at the basis. The calyx is 3-5 mm long, usually glabrous in the superior part, with triangular superior teeth, glabrous and slightly ciliated. The seeds are small, round and flattened. This species is common in Romania, being frequently found on the grassy hillocks, in the mountainous regions and on non-limestone soils.[16,17]

Isolation of essential oils

The aerial parts of the plant material were subjected to hydrodistillation, using a NeoClevenger apparatus, according to the method recommended by the European Pharmacopea. The obtained essential oils were stored at +4 °C until analysis.

Analysis of essential oils

The chemical composition of the volatile oil was established by GC-MS analysis with the help of an Agilent 6890N gas-chromatograph coupled to a mass selective detector (MSD) 5975 inert XL type. The conditions for chromatography were: column HP 5MS, mobile phase Helium – discharge: 1 mL/min, injector temperature: 250 °C, detector temperature: 250 °C, temperature regime from initial 40 °C (10 °C/min to 280 °C), injected volume: 0.1-0.3 µL, splitting ratio – 1:100.

Antibacterial activity

Testing the antibacterial activity has been carried out on 2 test microorganisms: *Streptococcus pyogenes* ATCC 19615 and *Pseudomonas aeruginosa* ATCC 27853 (from the collection of the Microbiology Laboratory, Al. I. Cuza University, Iasi), using both the diffusimetric method and the microplate method. The diffusion method used was Kirby-Bauer method, adopted by NCCLS (National Committee for Clinical Laboratory Standards, USA). As culture medium, Müller-Hinton in Petri plates has been used, uniformly dispersed in a layer of 4 mm thickness, pH 7.2-7.4, and premeasured. This medium has a nutritious value that allows an optimum development of a great variety of germs and does not contain bacterial inhibitors. Suspensions of the young bacterial cultures (18 hours old) were prepared. The plates were inoculated using a cotton swab immersed into the bacterial suspension. To absorb the inoculum, the plate has been left to rest for 3-5 minutes. We applied stainless steel cylinders on the medium surface using a sterile clip, afterwards distributing in each 200 µL of every dilution obtained by dissolving each type of oil in DMSO 10%. The plate has been incubated, lid down, at 37 °C for 16-18 hours; it is not indicated to put more than two plates on top of each other. For each individual sample, the antibacterial action of three volatile oils with the concentration of 100 µL/mL, 10 µL/mL and 1 µL/mL has been tested. The bacterial action has been assessed by measuring the diameter of the inhibition zone (mm) around the cylinders (containing the tested dilution) applied on the surface of the culture medium inoculated with the test microorganisms.

A modern method has been used to test the antimicrobial activity of the volatile oils – the microplates assay, using alamar blue or resazurine (modified resazurin microplate assay[18]). 96 well microplates were used, each containing 80 µl medium (MHB), 10 µL of diluted bacterial culture, 100 µL tested volatile oil in different concentrations and 10 µL resazurine, resulting in a total volume of 200 µL for each well. A few wells served as control, so they had no content of volatile oil. The microplates were incubated at 37 °C for 24 h. Bright pink colour is interpreted as positive bacterial growth, and the blue one indicates the absence of growth.

RESULTS AND DISCUSSION

Chemical composition of volatile oils

Analyzing the volatile oil from the five *Thymus pulegioides* populations from different areas, we identified a number of 64 compunds. In these samples, we noticed a series of differences, both qualitative and quantitative, regarding the chemical composition (Table 1).

Thus, in sample 1, 33 chemical compounds that represent 90.91% of the total column separated compounds have been identified. In this sample, the main constituents are: α-citral (16.40%), cis-nerolidol (15.81%), β-citral (12.49%), germacrene D (9.77%) and nerol acetate (7.20%). In sample 2, 29 compounds have been identified. The main compounds in this sample are: τ-cadinol (19.99%), limonene (13.50%) and β-bisabolene (4.94%). In the third analyzed sample, 32 chemical compounds, which represent 94.87% of the total column separated compounds, have been identified. In this case, the

main constituents are: cyclofenchone (18.79%), germacrene D (9.34%), τ-cadinol (8.31%), limonene (7.79%) and terpinen-4-ol (4.41%). The greatest number of chemical compounds has been identified for sample number 4, namely 35 compounds, compounds that represent 83.98% of the total column separated compounds. The main compounds in this sample are: germacrene D (12.25%), cyclofenchone (10.51%) and cis-nerolidol (6.38%). The smallest number of chemical compounds has been identified in sample 5, namely 27 compounds, representing 92.77% of the total column separated ones. In this sample, the main compounds are: carvacrol (39.35%), limonene (15.04%) and β-linalool (4.73%).

Table 1
Chemical composition of *Thymus pulegioides* volatile oils

Samples / Component	RT (min)	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)	Sample 4 (%)	Sample 5 (%)
α-Thujone	5.15		0.34	0.21		2.07
α-Pinene	5.26		2.19	0.76	0.65	1.33
Camphene	5.51		2.93	1.33	0.82	0.37
Octen-3-ol	5.89	1.41				
Isopentyl propionate	5.90		2.19	2.22	1.62	
3-Octanone	6.01		0.22			
β-Pinene	6.09	1.06		2.56		2.63
β-Myrcene	6.09				2.40	
3-Octanol	6.13		1.63			1.19
α-Phellandrene	6.33					0.33
δ-2-Caren	6.52					2.46
p-Cymene	6.63	0.13				
Limonene	6.72		**15.30**	**7.79**		**15.04**
Eucalyptol	6.76	0.31			2.30	
β-Ocimene	6.96	0.34				0.32
cis-Ocimene	6.97		3.06	2.45	2.31	
γ-Terpinene	7.16		0.34	1.12	0.50	7.57
trans-Sabinene hydrate	7.31					1.82
α-Terpinolene	7.61				0.20	
Camphor	7.75		1.74	2.30	1.84	
Ciclofenchone	7.74	0.74		**18.79**	**10.51**	
β-Linalool	7.78					**4.73**
Borneol	8.82		0.57	1.67	1.03	0.56
Terpinen-4-ol	8.98	0.75	0.76	**4.41**	0.96	2.73
α-Terpineol	9.16	1.19		0.65	2.29	0.52
Geranial	9.39	0.49				
cis-Geraniol	9.67	3.45	0.36	2.33	1.76	
β-Citral	9.88	**12.49**	0.77	2.19	1.34	
Nerol	10.01			3.47		
Lavandulol	10.03	3.54				
α-Citral	10.30	**16.40**	1.34	3.49	1.70	
Carvacrol	10.55				1.46	**39.35**
Thymol	10.54	0.35				
p-Thymol	10.69	0.48				
Nerolic acid	10.96	0.12				
α-Carene	11.39	0.34			1.57	
Terpinolene	11.44					0.43
Nerol acetate	11.54	**7.20**	1.05	0.48	1.06	
Lavandulol acetate	11.79	3.20	0.61			
β-Bourbonene	11.96	1.98	1.16		1.80	0.24
Methyl eugenol	12.10	0.12				
β-Caryophyllene	12.43	1.55	1.58	1.73	3.46	3.06
Humulene	12.88	0.16				
β-Cubebene	12.99				1.53	

Germacrene D	13.23	**9.77**	0.24	**9.34**	**12.25**	0.37
Bicyclgermacrene	13.44				1.84	
β-Bisabolene	13.49	2.21	**4.94**	3.87	1.92	
γ-Muurolene	13.62			2.95	0.16	
γ-Cadinene	13.63	0.15			2.63	
α-Cadinene	13.65		5.27			
δ-Cadinene	13.72	0.42		1.33		0.63
Calamenene	13.74		1.67			
Elemene	14.05			0.58	3.45	
cis-Nerolidol	14.21	**15.81**	1.88	1.92	**6.38**	0.67
Spathulenol	14.45			0.56	0.88	0.25
Caryophyllene oxide	14.55	1.21	0.68	0.83	1.07	1.16
1,4-Cadinadiene	14.94		3.09	1.61		
α-Gurjenen	15.00	0.20			0.24	
τ-Cadinol	15.37	0.79	**19.99**	**8.31**	4.80	0.53
α-Cadinole	15.41	0.88		1.63		0.37
α-Eudesmol	15.45				2.65	
α-Cedrene	15.80		1.58			
Eremophilene	15.81			0.63		
τ-Muurolol	16.11	0.95	0.11	1.36	2.60	2.04
Other compounds		9.09	22.41	5.13	16.02	7.23

Sample 1 – collected from Farcasa 1, sample 2 – Farcasa 2, sample 3 – Farcasa 3, sample 4 – Vama, sample 5 – Secuieni; RT – retention time

Regarding the phenolic monoterpens, thymol and carvacrol, specific to the *Thymus* genus plants, it has been noticed that they are very poorly represented in the analyzed populations, with the exception carvacrol, a ratio of 39.35% being identified in sample 5.

A general notice is that the monoterpens are the best represented in all the analyzed oil samples, according to previous studies,[9] except sample 2, in which an approximately equal share of sesquiterpens and monoterpens has been found.

Antibacterial activity of volatile oils

Regarding the antibacterial activity of the studied volatile oils, both the diffusion method and the microplates method have been used for a very precise analysis. Testing has been performed on 2 test microorganisms: *Streptococcus pyogenes* ATCC 19615 and *Pseudomonas aeruginosa* ATCC 27853, microorganisms from the collection mentioned above.

Evaluating the antibacterial activity by means of the diffusion method lead to the results presented in Table 2. By measuring the diameters of the inhibition zones (mm) around the stainless steel cylinders that contained different concentration of the tested oil samples, some differences between the two test microorganisms and the dilutions used has been noticed. Thus, in the case of *Streptococcus pyogenes* ATCC 19615, the diameters of the inhibition zones varied between 0-60 mm, and in the case of *Pseudomonas aeruginosa* ATCC 27853, between 0-35 mm. In the 5 volatile oil types belonging to different *Thymus pulegioides* populations, a higher antibacterial action of these oils in the case of the *Streptococcus pyogenes* species compared to *Pseudomonas aeruginosa* has been noticed, these results being confirmed by the diameters of the inhibition zones, greater in the first species.

To confirm the antibacterial action, another objective of the study was to establish the minimum inhibitory concentration (MIC), using the microplates method, in each type of volatile oil compared to the test microorganisms. Thus, the MIC for S*treptococcus pyogenes* is 1 µL/mL in volatile oil samples 1 and 2, and 100 µL/mL in samples 3, 4 and 5. For *Pseudomonas aeruginosa*, the MIC is 1 µL/mL in volatile oil samples 1 and 2, 10 µL/mL in sample 3 and 100 µL/mL in samples 4 and 5. The differences between the two tested bacteria may be related to the different ultrastructure of their cell wall (*Streptococcus pyogenes* – a Gram positive bacterium and *Pseudomonas aeruginosa* – a Gram negative bacterium), and also to the chemical composition of the tested volatile oils. The bactericide effect, much more obvious in the volatile oils towards *Streptococcus pyogenes* is probably due to some chemical compounds towards which this species manifests a raised sensibility.

Our studies confirm the fact that *Thymus pulegioide* volatile oils, which are rich in phenols and other compounds, inhibit the growth and development of the microorganisms.

Table 2
Antibacterial activity of volatile oils

Sample	Oil concentration (μL/mL)	Test microorganisms			
		Streptococcus pyogenes ATCC 19615		*Pseudomonas aeruginosa* ATCC 27853	
		Inhibition area			
		Microplate method	Diffusion method (mm)	Microplate method	Diffusion method (mm)
1	100	+++	60	+++	35
	10	+++	20	+++	11
	1	+++	10	+++	8
2	100	+++	66	+++	27
	10	+++	20	+++	11
	1	+++	10	+++	7
3	100	+++	10	+++	25
	10	---	0	+++	8
	1	---	0	--+	6
4	100	+++	50	+++	17
	10	--+	8	---	0
	1	--+	6	---	0
5	100	+++	60	+++	20
	10	---	0	---	-
	1	---	0	---	-
M.		---	-	---	-

Legend: + positive reaction; - negative reaction

CONCLUSION

Thymus pulegioides represents an important source of volatile oil. In the 5 analyzed populations, the monoterpenes are generally very well represented, except sample number 2. As a result of the antimicrobial analyses, the tested volatile oils, belonging to the 5 *Thymus pulegioides* populations, present a much more obvious antibacterial activity towards *Streptococcus pyogenes* species, as compared to *Pseudomonas aeruginosa*.

ACKNOWLEDGEMENTS: This work was supported by a grant of the Romanian Ministry of Education, CNCS – UEFISCDI, project number PN-II-RU-PD-2012-3-0307. We are also gratefully to project CERNESIM - POS CCE-O 2.2.1, SMIS-CSNR 13984-901, No. 257/28.09.2010-for the infrastructure used to complete this work.

REFERENCES

[1] R. Morales, in "The Genus *Thymus*", Taylor and Francis, London, 2002, pp. 1-44.
[2] V. Ciocarlan, "Flora ilustrata a Romaniei. Pteridophyta et Spermatophyta" [Illustrated Flora of Romania. Pteridophyta et Spermatophyta, in Romanian], Ed. Ceres, Bucuresti, 2009.
[3] M. Corticchiato, F. Tomi, A. F. Bernardini and J. Casanava, *Biochem. Syst. Ecol*., **26**, 8 (1998).
[4] Z. Kisgyorgy, K. Csedo, H. Horster, J. Gergely and G. Razc, *Rev. Med.* (*Targu-Mures*, *Romania*), **29**, 124 (1983).
[5] B. M. Lawrence, *Perfumer Flavorist*, **23**, 3 (1998).
[6] A. Schmidt, Doctoral Thesis, University of Hamburg, 1998.
[7] E. Stahl Biskup, *J. Essent. Oil Res*., **3**, 61 (1991).
[8] D. Mockute and G. Bernotiene, *Bio. Sis. Eco*., **29**, 1 (2001).
[9] E. Stahl Biskup, in "The Genus *Thymus"*, Taylor and Francis, London, 2002, pp. 75-124.
[10] T. Kowal and A. Kuprinska, *Herba Pol*., **25**, 303 (1979).

[11] M. Marino, C. Bersani and G. Comi, *J. Food Prot*., **62**, 9 (1999).
[12] R. R. Nelson, *J. Antimicrob. Chemother*., **40**, 2 (1997).
[13] C. Pina-Vaz, R. A. Goncalves, E. Pinto, S. Costa-de-Oliveira, C. Tavares *et al*., *J. Eur. Dermatol. Venereol*., **18**, 1 (2004).
[14] A. Smithpalmer, J. Stewart and L. Fyfe, *Lett. Appl. Microbiol*., **26**, 2 (1998).
[15] B. J. Juven, J. Kanner, F. Schued, and H. Weisslowicz, *J. Appl. Bacteriol*., **76**, 6 (1994).
[16] J. Jalas, "Flora Europaea", Cambrige University Press, 1972, vol. 3, pp. 172-182.
[17] M. Gusuleac, in "Flora Republicii Populare Romane" [The Flora of Romanian People's Republic, in Romanian], VIII, Academia RPR, Bucuresti, 1961, pp. 87-394.
[18] S. D. Sarker, L. Nahar and Y. Kumarasamy, *Methods*, **42**, 4 (2007).