ORIGINAL PAPER

THE STUDY OF ESSENTIALS OILS OBTAINED FROM *THYMUS PANNONICUS* L. - MICROBIOLOGICAL ASPECTS

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Received: 7 July 2018; **Accepted:** 9 July 2018; **Published:** 15 July 2018

Abstract: Essentials oils have been used over time in the food and cosmetics industry, but also in the medical and pharmaceutical industry. Environmental factors such as temperature, radiation and photoperiod play an extremely important role in the quantity and quality of volatile oils. It is also known that the vegetation stage can play an important role in the chemical composition of volatile oils. The purpose of this paper is to establish the antibacterial and antifungal activity of volatile oils of *Thymus pannonicus*, taking into account the ontogenetic stage in which the plants were collected, highlighting the compounds of therapeutic importance. To test the antimicrobial activity of essential oils two methods of work were used: Kirby-Bauer disc diffusion method and microplate method. The essential oils studies were tested on *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans*. It was find that all essential oils tested have antimicrobial activity at all stages of development tested. The maximum antimicrobial activity has been recorded for the oils extracted from individuals collected at the anthesis stage.

Keywords: volatile oils, composition, antimicrobial activity, thyme, ontogenetic stage.

1. Introduction

The species of the *Thymus* genus exhibit tonic, carminative, digestive, antitussive, expectorant properties (Mojab et al., 2008), which is why volatile oils have been extensively studied and tested on various microorganisms. Thus, essentials oil of *Thymus vulgaris* exhibits antifungal properties, being tested on *Aspergillus, Candida, Penicillium, Mucor, Cladosporium, Trichoderma, Chaetomium* (Segvic Klaric et al., 2006; Giordani et al., 2004; Faleiro et al., 2003). At present, there are numerous studies on the antibacterial activity of volatile oils belonging to the genus *Thymus* (Kowal and Kuprinska, 1979; Marino, 1999; Nelson, 1997; Pina-Vaz et al., 2004; Smithpalmer et al., 1998). Timol and carvacrol seem to play an important role in this. These phenolic compounds bind to the amino- and hydro-amino groups of proteins in the bacterial membrane, altering their permeability, thereby leading to battery death (Juven et al., 1994). According to studies conducted by Pina-Vaz C. et al. (2004), essential oil from *Thymus vulgaris*, *Thymus zygioides* ssp. *zygioides* and

Thymus mastichine, can be used for medicinal purposes. The antibacterial activity of the main components of volatile oil (carvacrol, timol, p-cimen and 1,8 cineol) and the possible interactions between these components were studied. Oils from *Thymus vulgaris* and *Thymus zygioides* have shown similar antibacterial activity, and higher than that of *Thymus mastichina*. Also, volatile oil of *Thymus vulgaris* was also tested on *E. coli* (Marino, 1999), demonstrating that *E. coli* cells are destroyed at a relatively low concentration of oil. Possible antimicrobial activities of volatile oil of *Thymus* have been investigated by Faleiro et al. (2003). The authors analyze the chemical composition and test the antimicrobial activity of oils obtained from *Thymus mastichina* ssp. *mastichina*, *Thymus camphoratus* and *Thymus lotocephalus*, species harvested in different areas of Portugal. The antimicrobial activity of these oils was tested on *Candida albicans, Escherichia coli, Listeria monocytogenes, Proteus mirabilis, Salmonella* spp. and *Staphylococcus aureus*. The studied *Thymus* species demonstrated antimicrobial activity, but the tested microorganisms exhibited different sensitivities. Also, this antimicrobial activity is due to several components of volatile oils. The antimicrobial properties of the *Thymus pubescens* and *Thymus serpyllum*, species harvested before and during flowering, have been studied by Rasooli and Mirmoreafa (2002). Also volatile oil extracted from *Thymus revolutus*, a species growing on the territory of Turkey, presents important antibacterial and antifungal activities.

2. Materials and Methods

2.1 Plant material

The plant material is represented by *Thymus pannonicus* ssp. *auctus* All. and *Thymus pannonicus* ssp. *pannonicus* (Lyka) Soo. collected from Dealul Șorogari, jud. Iași, in three phenophases of development: vegetative, anthesis and fructification.

Species identification was performed by Dr. Ioan Sârbu from Botanic Garden "Anastasie Fătu", Iași and by prof. Dr. Nicolae Ștefan, taxonomist at Faculty of Biology, University "Al. I. Cuza" University of Iași.

The identification of taxa has been done using the following papers: Flora Europaea, vol. 3 and Flora ilustrată a României - Pteridophyta et Spermatophyta (Ciocârlan, 2009). The collected material was registered and stored in „Alexandru Ioan Cuza" University's Herbarium from Iași (*Thymus pannonicus* ssp. *auctus – no. 182354, Thymus pannonicus* ssp. *pannonicus – no. 182353)*.

2.2 Isolation of essential oils

100 g dried plant were subjected to hydro-distillation, 3 hours, using a NeoClevenger apparatus, according to the method recommended by the European Pharmacopeia (1997). The obtained essential oils were stored at +4°C until analysis.

2.3 Antimicrobial activity methods

To test the antimicrobial activity of essential oils two methods of work were used: Kirby-Bauer disc diffusion method and microplate method. The essential oils studies were tested on *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans*.

2.3.1 Kirby-Bauer diffusion method

The sensitivity of microorganisms to the volatile oils under study was tested "in vitro" using optimal and standardized cultivation conditions (culture medium, inoculation, incubation etc.). Diffusion method Kirby-Bauer adopted by CLSI (Clinical Laboratory Standards International, 2009) in the US is the usual method, widely used in laboratories to

test a relatively small number of microbial strains with rapid growth. By submitting steel cylinders containing 50µl quantities of samples tested (volatile oil to be tested in various concentrations: 10%, 1% and 0.1%, oil was dissolved in 10% DMSO), on the surface of a solid medium inoculated with a microbial culture, active antimicrobial substance will diffuse into the environment, with a steady decline of the concentration gradient from the edge of the cylinder toward the periphery. After the incubation time two separate zones will appear: one in which microbial growth is inhibited by concentrations of the antimicrobial substance, and a zone where the concentration is too low to inhibit the growth.

The culture medium used is Mueller Hinton medium (for bacteria) and Sabouraud medium (for yeast), distributed in Petri dishes in a uniform layer thickness of 4 mm, a pH of 7.2 to 7.4 (for bacteria) and pH 6.5 (for yeast) measured before pouring into plates. These medium have nutritional value which allows optimum development of a wide variety of germs and contains no inhibitors of bacterial substances.

2.3.2 Minimum inhibitory concentration

The second method used is microplate method (Sarker et al., 2007). We used 96-well microplate, each containing 80 ml culture medium, 10 µl of diluted bacterial culture, 100 µl essential oil to be tested in different concentrations (10%, 1% and 0.1%; oil was dissolved in 10% DMSO) and 10 µl resazurine, resulting in a total volume of 200 µl per well. Microplates were incubated at 37°C for 24 hours. Of course, each plate contained wells and representing control (represented by DMSO). The colour changes were then evaluated visually. Thus, growth and development of microorganisms was indicated by changing colour from dark blue to purple. MIC (minimum inhibitory concentration) is the lowest concentration at which the colour changes.

2.4 Statistical analysis

For both working methods, positive controls (kanamycin for *Staphyloccocus aureus* and *Escherichia coli* and Nystatin for *Candida albicans*) and negative controls (distilled sterile water) were used. Also, all the determination was performed in triplicates and the results presented in tables representing an average.

3. Results and discussions

3.1 Testing antibacterial activity of essentials oils used on *Staphylococcus aureus*

The results of testing the antibacterial activity of volatile oils from the two subspecies of Thymus pannonicus are shown in **Table 1.** As can be seen, all the oils tested show antibacterial activity, differing only in MIC (minimum inhibitory concentration). The largest inhibition zone (17 mm) was recorded for the volatile oil of *Thymus pannonicus* ssp. *pannonicus* - vegetative stage. The main compounds for this oil are germacren D, farnesol and trans nerolidol. Germacren D is a known compound due to its antimicrobial properties. Farnesol is generally known as a pesticide and pheromone (Boz et al., 2016) In the literature, there are studies on the antibacterial activity of volatile oils of *Thymus pannonicus* (anthesis stage) (Maksimović, 2008), but not on the activity of volatile oils from the two subspecies of the genus.

3.2 Testing the antibacterial activity of essentials oils analyzed for *Escherichia coli*

Data on antibacterial activity of volatile oils derived from *Thymus pannonicus* ssp. *auctus* and *Thymus pannonicus* ssp. *pannonicus* collected in various phenophases are shown in **Table 2.** As can be seen, the minimal inhibitory concentration (MIC) for the tested concentrations is 0.1% with the

exception of volatile oils collected from plants in the fructification stage, where the MIC is 1%. The largest inhibition zone (11 mm) was found in the *auctus* ssp, at the anthesis stage and at a concentration of 10%. The main compounds of this oil are germacren D, farnesol, trans nerolidol and terpinyl acetate (Boz et al., 2016).

3.3 Testing the antifungal activity of essentials oils analyzed for *Candida albicans*

Data on the antifungal activity of volatile oils from *Thymus pannonicus* ssp. auctus and *Thymus pannonicus* ssp. *pannonicus* collected in various phenophases are shown in **Table 3.** As can be seen, the MIC for the tested concentrations is 0.1% with the exception of volatile oils collected from plants in the fructification stage, where the MIC is 1%. The largest inhibition zone (10 mm) is observed for the volatile oil extracted from the *Thymus pannonicus* ssp. auctus (anthesis stage) at a concentration of 10%. The main compounds of this oil are germacren D, farnesol, α terpinyl acetate and trans-nerolidol (Boz et al., 2016).

The results obtained show that all the oils tested show antimicrobial activity, differing only in MIC. Generally, the oils obtained from plants in the vegetative stage and the anthesis stage has a higher activity. From the available literature, we have not identified studies on the chemical composition and antimicrobial activity of volatile oils from the 2 subspecies of *Thymus pannonicus*. There are, however, studies on volatile oil of *Thymus pannonicus*. Thus, Maksimovic and colleagues identified a total of 33 chemical compounds in the volatile oil of *Thymus pannonicus*, collected from Serbia, in 2008, the main ones being geranial (41.42%) and neral (29.61%). These compounds have not been identified in volatile oil from species collected in Romania (Boz et al., 2009).

Table 1. Testing of antibacterial activity of volatile oils of *Thymus pannonicus* ssp. *auctus* and *Thymus pannonicus* spp. *pannonicus*, collected in various phenophases, on *Staphylococcus aureus*

Sample	**Oil concentration %**	***Staphyloccocus aureus***	
		Diffusimetric method Inhibition zone - mm	**Microplates method** + positive reaction, - negative reaction
Thymus pannonicus ssp. *auctus* – vegetative stage	10	12	+++
	1	8	+++
	0.1	6	+++
Thymus pannonicus ssp. *auctus* – anthesis stage	10	10	+++
	1	6	+++
	0.1	0	++-
Thymus pannonicus ssp. *auctus* – fruit stage	10	8	+++
	1	0	++-
	0.1	0	---
Thymus pannonicus ssp. *pannonicus* - vegetative stage	10	17	+++
	1	10	+++
	0.1	6	+++
Thymus pannonicus ssp. *pannonicus*– anthesis stage	10	12	+++
	1	6	+++
	0.1	5	+++
Thymus pannonicus ssp. *pannonicus*– fruit stage	10	12	+++
	1	5	+++
	0.1	0	---

Table 2. Antibacterial activity testing of volatile oils of *Thymus pannonicus* ssp. *auctus* and *Thymus pannonicus* ssp. *pannonicus* collected in various phenophases on *Escherichia coli*

Sample	**Oil concentration %**	***Escherichia coli***	
		Diffusimetric method Inhibition zone - mm	**Microplates method** + positive reaction, - negative reaction
Thymus pannonicus ssp. *auctus* – vegetative stage	10	10	+++
	1	7	+++
	0.1	6	+++
Thymus pannonicus ssp. *auctus* – anthesis stage	10	11	+++
	1	7	+++
	0.1	5	+++
Thymus pannonicus ssp. *auctus* – fruit stage	10	9	+++
	1	5	+++
	0.1	0	---
Thymus pannonicus ssp. *pannonicus* - vegetative stage	10	10	+++
	1	8	+++
	0.1	6	+++
Thymus pannonicus ssp. *pannonicus*– anthesis stage	10	9	+++
	1	6	+++
	0.1	5	+++
Thymus pannonicus ssp. *pannonicus*– fruit stage	10	10	+++
	1	6	+++
	0.1	0	---

Table 3. Antifungal activity testing of volatile oils of *Thymus pannonicus* ssp. *auctus* and *Thymus pannonicus* ssp. *pannonicus* collected in various phenophases on *Candida albicans*

Sample	**Oil concentration %**	***Candida albicans***	
		Diffusimetric method Inhibition zone - mm	**Microplates method** + positive reaction. - negative reaction
Thymus pannonicus ssp. *auctus* – vegetative stage	10	8	+++
	1	7	+++
	0.1	6	+++
Thymus pannonicus ssp. *auctus* – anthesis stage	10	10	+++
	1	7	+++
	0.1	5	+++
Thymus pannonicus ssp. *auctus* – fruit stage	10	9	+++
	1	5	+++
	0.1	0	---
Thymus pannonicus ssp. *pannonicus* - vegetative stage	10	8	+++
	1	8	+++
	0.1	6	+++
Thymus pannonicus ssp. *pannonicus*– anthesis stage	10	7	+++
	1	6	+++
	0.1	5	+++
Thymus pannonicus ssp. *pannonicus*– fruit stage	10	7	+++
	1	6	+++
	0.1	0	---

Other scientists identified large amounts of thymol (25-41%) and p-cimen (17-38%) in the volatile oil of *Thymus pannonicus* (Pluhar et al., 2007). The antimicrobial activity of the oil was evaluated using agar disc diffusion and broth microdilution method against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli*, two strains of *Klebsiella pneumoniae* and two strains *of Candida albicans*. The essential oil exhibited antimicrobial activity to varying degrees against all tested strains (Maksimović et al., 2008).

Conclusions

In conclusion, we can say that all essential oils tested have antimicrobial activity at all stages of development tested, differing only in MIC. In most of the variants tested, intensification of antimicrobial activity occurs with an increase in oil concentration of up to 10%. Considering the plant development phase, we find that for the most part the maximum antimicrobial activity has been recorded for the oils extracted from individuals collected at the anthesis stage, also knowing that the flow of volatile compounds is intensified in order to ensure attraction of pollinators.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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.

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