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Study on chemical composition and antifungal activity of essential oils obtained from representative species belonging to the Lamiaceae family

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ABSTRACT

The main objective of the present study is establishing the chemical composition and minimum concentration of essential oil (EO) extracted from *Thymus vulgaris* L., *T. serpyllum* L., and *Satureja montana* L., which induce the mycelial growth inhibition of *Verticillium dahliae* and *Penicillium aurantiogriseum* fungi. *In vitro* testing on CYGA (chloramphenicol-yeast-glucose-agar) medium, with additional oil at 0.25, 0.5, 1, 5, 10 and 15 mg/L concentrations and inoculated with harvested plugs from a young mycelium, pointed out a different reaction of the fungus depending on the oil types and concetrations used. The minimum concentration that ensure inhibiting of mycelial growth for *V. dahliae* with significant differences compared to control is 0.25 mg/L for all types of EO. *P. aurantiogriseum* proved sensitivity at 0.25 mg/L for *T. vulgaris*, and *S. montana* EOs and 0.5 mg/L for *T. serpyllum* EO.

Keywords: natural product; antifungal capacity; herbs; carvacrol; ecological fungicide; GC/MS analysis

The concern for the quality of primary products with green origins is increasing nowadays (Abadias et al. 2008). Avoiding chemical compounds in plant treatments is the main path to ensure products uncontaminated with fungicides and pesticides residue. Therefore, research is carried out for alternative solutions of crops protection, friendly for the environment and safe for human consumption (Gan-Mor et al. 2011). It is known that plants from the Lamiaceae family have the ability to protect themselves against potential pests by synthesising some compounds (Bakkali et al. 2008). Among different phytocompounds the essential oils (EOs) are one of the most promising groups of natural products for ensuring a safer solution with guaranteed anti-pest and anti-microbial effects (Zoubiri and Baaliouamer 2010, Christaki et al. 2012, Singh et al. 2012).

The species *Thymus vulgaris*, *T. serpyllum*, and *Satureja montana* can be found in spontaneus flora from the Mediteraneean area and also in moderate European climate and it is presently seeded in extended areas, especially for the pharmaceutical industry. These species are cultivated for their aerial parts and are processed only dried to obtain volatile oil also known as EO. The EO content can vary, depending on sowing and climate conditions, and it is recommended that harvesting should start before the blooming phase, when the volatile oil content is at its peak (Muntean 2007).

The novelty of our study consists in demonstrating of the antifungal capacity of the Lamiaceae EOs on fungi such as *Verticillium dahliae*, and *Penicillium aurantiogriseum*.

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V. dahliae is a soil-born pathogen responsible for wilt diseases for many economically important crops and the problematic aspects are raised from two points of view (1) currently, there are no fungicides available to cure plants once they are infected with *Verticillium* and, (2) there are no unique symptoms that belong to all plants infected by this fungus (Fradin and Thomma 2006). Till now extracts from Canada milk vetch showed an ecological alternative from the *Verticillium* attack (El Hadrami et al. 2011).

P. aurantiogriseum is one of the most widespread moulds, resistant to drying and preservatives therefore being responsible for the degradation of grains during the post-harvest time along with other fungus species like *Aspergillus* sp. and *Fusarium* sp. (Magan et al. 2003). Studies of recent years revealed that *P. aurantiogriseum* can produce secondary metabolites such as roquefortine C and D with neurotoxic effects (Kozlovsky et al. 2009).

V. dahliae and *P. aurantiogriseum* are hard to combat even with synthetic anti-fungal substances. In this terms it is very important to search and to demonstrate the antifungal capacity of EOs from different plants against problematic fungus species. The main objectives of our research consisted in the evaluation of the chemical composition and assessment *in vitro* of antifungal potential of EOs obtained from *T. vulgaris*, *T. serpyllum* and *S. montana* cultivated in the western part of Romania.

MATERIAL AND METHODS

Isolation of EOs. *T. vulgaris* from Agrosem, and *T. serpyllum* and *S. montana* seeds from the Semillas Cantueso, Cordoba, Spain, were sown in a temperate climate zone, near Timisoara, Romania. The harvest of herbs took place in 2014 during the blooming period after a sunny period of days because sun light has a positive influence on the synthesis of the volatile oil, and other active substances. At the time of harvest the studied plants were in the 4^{th} year of vegetation. The fresh herbs were dried in a room with no sunlight access at a temperature between 20–22°C. The EOs were obtained through hydrodistillation using a volatile oil distilling Clevenger equipment at atmospheric pressure until no more EO was obtained. The extractions were performed at least three times and the mean values were reported. The extracted EOs were stored at +4°C until analysis.

Gas chromatography-mass spectrometry identification. The composition of EOs was determined using gas chromatography/mass spectrometry (GC/MS) analysis. Agilent Technology 7820A coupled with mass spectrometer MSD 5975 and equiped with a capillary column DB 5: (30 m × 250 µm × 0.25 µm, AGILENT, California, USA) was used. The carrier gas was helium with a constant flow of 1 mL/min. In order to separate the compounds, the following GC oven program was used: 40°C for 1 min, 5°C/min to 210°C for 5 min. The injector and ion source temperatures were 250°C and 150°C, respectively. The injection volume was 1 µL with a split ratio 1:20. The NIST (National Institute of Standards and Technology) spectra library has been used to identify the volatile compounds.

Bioassay of mycelial growth inhibition (MGI). The fungal cultures used in this study were provided by microbial collection of Microbiology Department of the Faculty of Horticulture and Forestry Timisoara. The *V. dahliae* strain was isolated from sea bucktorn, preserved at 4°C on PDA (potato dextrose agar) medium with Va09-13 index (Cotuna et al. 2014). The *P. aurantiogriseum* strain was isolated from the fungal microbiota of the wheat seeds preserved on PDA, at 4°C, with Lv07-11 index (Alexa et al. 2012). The method used to point out the inhibition of the mycelium was the poisoned food tehnique (Perrucci et al. 1994). First the fungi young cultures were obtained on CYGA (chloramphenicol-yeast-glucose-agar, SIGMA, Saint Louis, USA) by spread techniques with a spore suspension in melted agar 0.2% + TWEEN 80, 0.05%. After 4 days of growth in dark and constant temperature, we harvested plugs of Ø 8 mm from active mycelia and put them on CYGA medium amended with *T. vulgaris*, *T. serpyllum* and *S. montana* EOs at the following concentrations (v/v): 0.25, 0.5, 1, 5, 10 and 15 mg/L and 0 for control. Thiophanate-methyl, a commercial agricultural fungicide, was used as negative control for *Penicillium* and for *Verticillium* too. Experiments were conducted twice, each Petri plate containing EOs augmented medium, at different concentrations were inoculated with two plugs from young mycelia. After inoculations plates were kept in dark at 22 ± 2°C. After 5 days the radial mycelia growth was measured at two perpendicular diameters. The average of the readings was reduced by 8 mm representing the plug diameter. The minimum growth inhibition, was converted into percentage (MGI%), in relation to the control by using the for-

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mula C-Tx100/C (Riccioni and Orzali 2011) where C and T are the means of radial growth in control and EOs-treatment respectively, measured for each fungus from two experiments.

Statistical analysis. Analysis of variance (one way ANOVA) was performed on the data recorded for each fungus using statistical data analysis, from EXCEL, 2010 version (USA). The significance of the differences between the averages of EOs treatments and the control was evaluated using *t*-test at significance level $P < 0.05$.

RESULTS AND DISCUSSION

The chemical composition of EOs. In Table 1 we can find the chemical composition of the EOs, extracted from the three studied species. We took

Table 1. The major chemical compounds of the three studied *Thyme* spp. essential oils

Compound	Kovats retention index	*Satureja montana*	*Thymus serpyllum*	*Thymus vulgaris*
α-thujene	925	1.45	1.94	2.75
α-pinene	932	0.72	1.40	2.19
Camphene	946	0.24	0.64	1.68
2-β-pinene	974	0.17	0.36	0.57
1-octen-3-ol	977	0.88	0.96	0.81
β-myrcene	988	0.95	2.41	2.74
1-phellandrene	999	0.25	0.35	0.30
α-terpinene	1013	2.00	3.58	2.93
p-cymene	1018	12.68	16.16	32.92
D-limonene	1021	–	1.12	1.34
cis-β-ocimene	1023	0.45	–	–
1,8-cineole	1022	–	1.05	1.44
γ-terpinene	1044	3.51	23.85	14.31
Sabinene hydrate	1051	0.89	1.21	0.45
Linalool	1074	1.09	3.39	2.03
Camphor	1143	–	0.68	0.37
Borneol	1164	1.02	0.80	1.14
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)	1176	0.65	0.56	0.57
4.4-Dimethyl-1,5-cyclohexadienecarbaldehyde	1253	0.89	–	–
Citral	1270	–	0.58	–
Bicyclo[2.2.1]heptan-2-ol. 1,7,7-trimethyl acetate	1284	–	0.35	–
Endobornyl acetate	1285	–	–	0.44
Thymol	1291	0.36	6.31	3.33
Phenol, 5-methyl-2-(1-methylethyl), Carvacrol	1310	60.48	22.22	19.98
α-copaene	1376	0.23	–	–
Trans-caryophyllene	1517	3.12	4.48	1.97
Geraniol formate	1559	–	0.37	–
γ-cadinene	1568	0.91	0.47	–
β-bisabolene	1604	0.59	–	–
δ-cadinol	1611	0.44	–	–
δ-cadinene	1619	0.84	0.28	0.26
Caryophyllene oxide	1671	0.21	0.67	0.48
α-cadinol	1715	–	0.32	–
Total (%)		94.99	96.51	95.00

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under consideration the chemical compounds that were found in a quantity over 0.2% from the total amount. In the *T. vulgaris* EO we identified 81 compounds, of which 25 major compounds (over 0.2%) totalling over 95% from the total compounds. In *T. serpyllum* EO we found 72 compounds identified out of which 27 were major compounds (96.51%) while in *S. montana* EO we identified 67 compounds, 24 among them being in a major proportion (94.99%).

Carvacrol, a monoterpene phenol derivative represents the compound found in the largest amount in the EO extracted from *S. montana* (60.48%) while also being found in considerate quantities in the other 2 species studied, *T. vulgaris* (22.22%) and *T. serpyllum* (19.98%). The results obtained are similar to the data from the literature mentioning carvacrol as major component in *S. montana*, and thymol as being a compound in high content in *T. vulgaris* EO, (Baranauskiene et al. 2003, Porte and Godoy 2008, Schmidt et al. 2012). Fraternale et al. (2007) mentioned a content of 9.92% of thymol in *S. montana* EO while Muntean (2007) reports thymol under 10% in *T. serpyllum* EO. The content of volatile compounds varies with species, site of cultivation and the time of harvest. Therefore, Imelouane et al. (2010), identifies camphor as being the major compound (39.39%) in *T. vulgaris* EO cultivated in Morocco. The results obtained in this study show that *p*-cymene is the main compound in *T. vulgaris* EO (32.92%), and also, in high concentrations over 10% in the other analysed species, respectivelly, *T. serpyllum* (16.16%) and *S. montana* (12.68%). As well, *γ*-terpinene is a compound found in high quantities in *T. vulgaris* (14.31%) and *T. serpyllum* (23.85%), but was found in low quantities in *S. montana* (3.51%).

Bioassay of MGI for *V. dahliae*. The inhibition of the mycelium growth of *V. dahliae* is obvious for *T. vulgaris* EO at 0.25 mg/L registering 10% MGI value (Figure 1a). At a higher concentration of *T. vulgaris* EO the MGI value is 0. Similar studies were carried out to observe the *T. vulgaris* oils' effect. Concentration below 0.5 mg/L of *T. vulgaris* EO was sufficient for inhibiting growth of some pathogenic seed borne fungi (Riccioni and Orzali 2011). *T. serpyllum* EO has a less inhibitive effect over the fungus growth compared to *T. vulgaris* EO. Therefore at 0.25 mg/L concentration, MGI is to 30%, its value dropping with the rise of oil concentration to 8% and 3% MGI value. *S. montana* EO showed a similar effect to the *T. vulgaris* EO, the total mycelium growth inhibition being confirmed at the concentration of 0.5 mg/L.

The ANOVA test was applied only for the growth samples that recorded an MGI value under 100%, and confirmed the differences compared to control as being statistically significantly negative for significance level $P < 0.05$.

Bioassay of MGI for *P. aurantiogriseum*. As we can observe in Figure 1b, *P. aurantiogriseum* is highly sensitive to the *T. vulgaris* EO, the MGI values being 0 at all the tested concentrations. Also, we can see that the mycelium is less affected in the presence of *T. serpyllum* EO at the 0.25 mg/L, MGI has a value of 99%. At 0.5 mg/L *T. serpyllum* EO the MGI value drops at 22%. The different effect of the *Thymus*

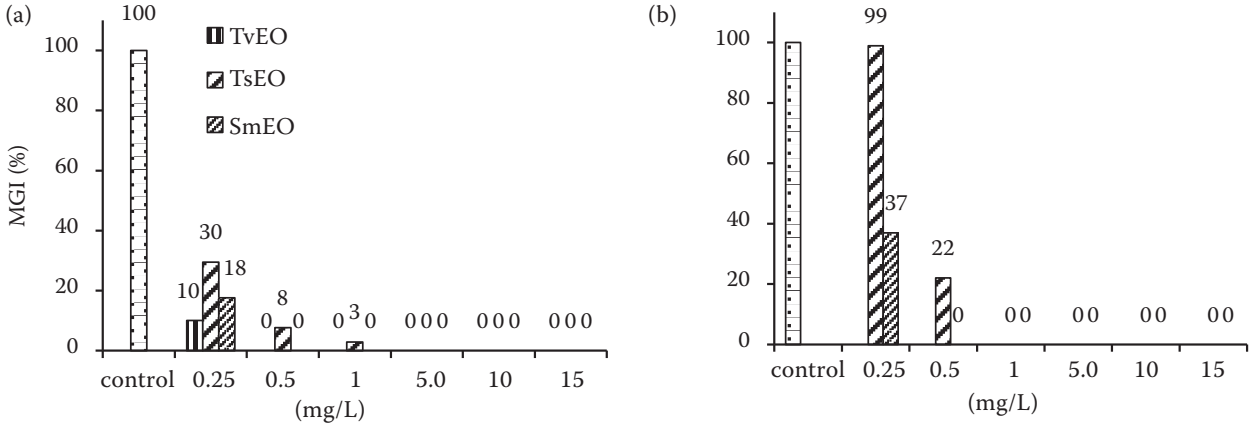

Figure 1. Mycelial growth inhibition (MGI) for (a) *Verticillium dahliae* and (b) *Penicillium aurantiogriseum* in presence of different essential oils (EOs) concentration. TvEO – *Thymus vulgaris* EO; TsEO – *T. serpyllum* EO; SmEO – *Satureja montana* EO

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spp. EOs can be explained by the various chemical composition that changes the anti-fungal properties as shown in other studies made on *T. zygis* and *T. hyemalis* EOs (Rota et al. 2008).

In case of *S. montana* EO mycelium growth inhibition starts at 0.25 mg/L having the MGI value of 37%. Higher concentrations determine a total inhibitory effect, the MGI value becoming 0. Despite the fact that the *S. montana* EO has the highest carvacrol concentration, the inhibitory effect of the *P. aurantiogriseum* mycelium growth is not caused only by its presence. Previous researches have shown that the anti-fungal effect is not caused only by one major compound, also the synergy of the other compounds in smaller amounts can have the same effect (Nakats et al. 2000). The proportion of γ-terpinene compound found in *T. serpyllum* EO over 23%, indicates the fungus tolerance to this compound. Furthermore, Dorman and Deans (2000), argue that γ-terpinene si p-cymene have limited anti-fungal capacity.

Analysis of EO obtained from *S. montana*, *T. vulgaris* and *T. serpyllum* by GC-MS demonstrated that carvacrol, γ-terpinene, p-cymene and thymol, are the main compounds from *Thymus* spp. cultivated in West of Romania. The EO extracted from plants belonging to *Thymus* genus have a high anti-fungal capacity and can be used as ecological fungicide against *V. dahliae* and *P. aurantiogriseum.* The anti-fungal activity is connected to the presence of an aromatic nucleus which contains a polar phenolic group from the carvacrol structure. The fungus' sensitivity varies according to the type of EO. Therefore, for *V. dahliae* the minimum concentration which provides mycelial growth inhibition with significant differences compared to control is 0.25 mg/L for all types of EO while *P. aurantiogriseum* proved sensitivity at 0.25 mg/L for *T. vulgaris*, and *S. montana* EOs and 0.5 mg/L for *T. serpyllum* EO for a guaranteed period of 5 days.

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