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Original scientific paper

Antifungal activity of six plant essential oils from Serbia against *Trichoderma aggressivum* f. *europaeum*

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SUMMARY

Six essential oils (EOs) extracted from plants originating in Serbia were assayed for inhibitory and fungicidal activity against a major fungal pathogen of button mushroom causing green mould disease, *Trichoderma agressivum* f. *europaeum*. The strongest activity was demonstrated by the oils of basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.). Medium antifungal activity of St. John's wort (*Hypericum perforatum* L.) and walnut [*Juglans regia* (F)] oils was also recorded. Oils extracted from yarrow (*Achillea millepholium* L.) and juniper (*Juniperus communis* L.) exhibited the lowest activity. Peppermint oil showed fungicidal effect on the pathogen, having a minimum fungicidal concentration of 0.64 µl ml^{-1}. The main components of peppermint essential oil were menthone (37.02%), menthol (29.57%) and isomenthone (9.06%).

Keywords: Essential oils; Trichoderma; Edible muhsrooms

INTRODUCTION

Production of button mushroom [*Agaricus bisporus* (Lange) Imbach] is affected by several diseases, resulting in lower yield and quality of marketable fruiting bodies. The major fungal pathogen, *T. aggressivum* f. *europaeum* Samuels & W. Gams, has been transmitted from the British Isles to many European countries including Serbia (Kosanović et al., 2013). The pathogen causes green mould disease, accounting for losses ranging between 30% and 100% (Grogan, 2008). Green mould disease of mushrooms is characterized by dense white mycelia of fast-growing colonies on casing or compost that changes colour into green after extensive sporulation. Spots on fruiting bodies of *A. bisporus* are early and accompanying symptoms. In serious outbreaks, no fruiting bodies are produced (Seaby, 1996).

A common method of disease control in mushroom farms is treatment of casing soil with disinfectants and fungicides. Many pathogens develop resistance to fungicides, and ongoing EU pesticide reviews have resulted in withdrawal of approval for many chemicals, mainly in the group of benzimidazoles (Fletcher et al., 1989; Grogan, 2008). As a result, only a few chemicals

are currently available, and effective alternatives are required. The fungicides that are officially recommended in mushroom industry are: prochloraz in the EU countries, and chlorothalonil, thiabendazol and tiophanate-methyl in North America (Beyer & Kremser, 2004; Grogan, 2008). Initially, benzimidazole fungicides applied to spawn in the US had provided good control of *T. aggressivum* f. *aggressivum*, but the problem of occurring resistant isolates has emerged nevertheless, leaving improved hygiene as the only option for control of this pathogen (Romaine et al., 2005). The most effective fungicide in mushroom disease control is prochloraz (Grogan, 2008). Prochloraz is still effective against fungal pathogens in Serbia (Potočnik et al. 2008, 2009, 2010; Kosanović et al., 2013), though decreased sensitivity of pathogenic fungi of the genera *Lecanicillium* and *Cladobotryum* has been already reported in Spain and Great Britain (Gea et al., 2005; Grogan, 2008).

In the course of evaluation of alternative means, antimicrobial properties of essential oils (EOs), as well as their components, have been demonstrated. Oils with very strong activity could be proved as promising agents in future extensive research and *in vivo* examination. Oils of oregano (*Origanum vulgare* L.) and common thyme (*Thymus vulgaris* L.) have shown very high *in vitro* activity against *T. aggressivum* f. *europaeum*, *T. harzianum* Rifai, and *T. atroviride* P. Karsten, while oils and components of peppermint (*Mentha piperita*L.) have been found to inhibit the growth of *T. viride* Tul. (Soković & van Griensven, 2006). Addition of tea tree oil [*Melaleuca alternifolia* (Maiden & Betche) Cheel] to oyster mushroom substrate or button mushroom casing has resulted in considerable *in vivo* inhibition of *T. harzianum* (Angelini et al., 2008; Kosanović et al., 2013).

Peppermint, basil, yarrow, St. John's wort, walnut, and juniper have been acknowledged as herbs with a plenty of pharmacological properties. These plants have been used in herbal medicine, and some of them for their flavours and as antimicrobial agents (Soković & van Griensven, 2006). Therefore, these plants growing in Serbia have been chosen for *in vitro* testing of antimicrobial activities of their oils against *T. aggressivum* f. *europaeum* in an attempt to promote their use as alternative products for disease control.

MATERIAL AND METHODS

Plant material and preparation of essential oils

Six EOs were provided from different plants growing in Serbia. Peppermint herbal tea (*Mentha piperita* L.) was purchased from "Bilje Borča" d.o.o.; walnut leaves [*Juglans regia* (F)] were collected in May of 2011 (Zemun, Belgrade); yarrow flowers and leaves (*Achillea millepholium* L.) were collected in May of 2010 (Divčibare); basil flowers and leaves (*Ocimum basilicum* L.) were collected in July of 2011 (Mali Mokri Lug, Belgrade); St. John's wort plants (*Hypericum perforatum* L.) were collected in May of 2011 (Ljubovija) and juniper cones (*Juniperus communis* L.) were collected in May of 2011 (Ljubovija).

All plant material was air-dried in shade at room temperature for 20 days, and then subjected to hydro-distillation for 2.5 h, in a Clevenger type apparatus. The obtained essential oils were dried over anhydrous sodium sulphate and preserved in sealed vials at 4°C until further analysis.

Test organism and inoculum preparation

The isolate of *Trichoderma aggressivum* f. *europaeum* T77 used in the study was obtained from an *A. bisporus* substrate with green mould disease symptoms collected from a Serbian mushroom farm (locality Barajevo-Lisovići) in 2010 and previously identified (Kosanović et al., 2013). The isolate was maintained on Potato Dextrose Agar (PDA) medium at 20°C for 72 hours. Conidia were harvested by flooding the plates with 10 ml of sterile distilled water and Tween 20 (v/v 0.01%), followed by filtration through a double layer of cheesecloth. Conidial suspension was prepared daily in sterile saline and adjusted to a concentration of approximately 10^6 conidia ml^{-1}.

Antifungal activity of essential oils in vitro

Antifungal activity was tested on PDA medium in glass Petri plates (R=100 mm).The medium was inoculated with the investigated fungi by pipetting 20 µl of conidial suspension into each well cut at the centre of the plate (R=10 mm). Inoculum was then exposed to the volatile phase of EOs for 48 h at 20°C. The oils were applied as a single drop onto the inner side of each plate cover at concentrations of 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 µlml^{-1} of air inside Petri plates using a micropipette. The bottom of each plate was immediately placed upon the cover. The plates were sealed with parafilm to prevent gas exchange with the outside environment. Oil concentrations that completely inhibited bacterial growth after two-day-exposure at 20°C were considered to be fungistatic and the lowest of these concentrations was determined as Minimum Inhibitory Concentration (MIC). The plates were then opened and ventilated in a laminar flow hood for 30 min in order to remove volatiles and determine fungicidal effect. Oil concentrations were considered fungicidal if

no fungal growth was observed two days after ventilation. The lowest concentration that had fungicidal effect was defined as Minimum Fungicidal Concentration (MFC) (Tanović et al., 2006). Four replicates per treatment were used and the experiment was repeated twice.

Essential oil analysis

Analyses of all essential oils were performed by gas chromatography (GC) using two detector types. An Agilent GC (7890A model) was equipped with a split/splitless injector, a flame ionization detector (FID) and a HP-5 capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness). Injector and detector temperatures were set to 250 and 300 ^{0}C, respectively, while the nitrogen flow rate was 1 ml/min. Column temperature was linearly programmed from 50 to 250 ^{0}C at 4^{0}C/min and held for 10 minutes. Analyses with a mass spectrometer (MS) as the detecting device were conducted using a Varian CP–3800/Saturn 2200 model. Both the injector temperature and column temperature programmes were the same as for GC-FID analysis, while separation was performed using Agilent DB-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas (1 ml/min), and the ion trap and transferline temperatures were set to 250 ^{0}C and 280 ^{0}C, respectively. The mass detector was operated in the electron impact (EI) mode (70 eV; 40-600 m/z range). In both cases, essential oil solutions in n-hexane (1%) were injected in split mode (1:20).

In order to determine the retention indices (RI), a mixture of n-alkanes (C_6-C_{28}) was analysed by both GC-FID and GC-MS under the same conditions as essential oils.

Identification of essential oil components was performed using both Wiley 7.0 mass spectral library and the obtained RI data, while quantitative data were expressed as area percent obtained by GC-FID analysis.

RESULTS AND DISCUSSION

Analysis of essential oils

The yields of the essential oils isolated from mint, basil, John's wort, walnut, yarrow and juniper were 1.9%, 0.76%, 0.17%, 0.23%, 0.37% and 2% (v/w in dry matter), respectively.

As only peppermint oil exhibited the lethal effect on the pathogen (Table 1), it was chosen for chemical composition analysis (Table 2). As a result of that analysis, thirty two components of peppermint essential oil were identified, and they accounted for 99.07% (v/w) of total oil mass.

Table 1. Effective concentrations of essential oils (μl ml^{-1} of air) against *Trichoderma aggressivum* f. *europaeum* T7

Essential oils	MFC[a]	MIC[b]
Peppermint (*Mentha piperita* L.)	0.64	0.04
Common walnut [*Juglans regia*(F)]	>0.64	0.16
Yarrow (*Achillea millefolium*L.)	>0.64	0.64
Basil (*Ocimum basilicum* L.)	>0.64	0.02
Common St. John's wort (*Hypericum perforatum* L.)	>0.64	0.08
Common juniper (*Juniperus communis* L.)	>0.64	>0.64

[a]Minimal concentration of oil showing lethal effect on the pathogen (Minimum Fungicidal Concentration)
[b]Minimal concentration of essential oil causing complete inhibition of bacterial growth after seven-day exposure (Minimum Inhibitory Concentration)

Table 2. Chemical composition of peppermint (*Mentha piperita* L.) essential oil

N^o	Components	RI*	Content (%)
1	α-pinene	932	0.48
2	sabinene	970	1.26
3	β-myrcene	986	0.49
4	δ-3-carene	1010	0.09
5	α-terpinene	1018	0.09
6	p-cymene	1022	1.20
7	1,8-cineole	1025	4.52
8	(Z)-β-ocimene	1030	0.40
9	(E)-β-ocimene	1040	0.09
10	γ-terpinene	1052	0.17
11	trans-sabinene hydrate	1060	0.65
12	linalool	1096	0.43
13	menthone	1150	37.02
14	isomenthone	1160	9.06
15	neo-menthol	1169	2.52
16	menthol	1173	29.57
17	α-terpineol	1183	0.28
18	pulegone	1234	0.78
19	piperitone	1249	1.38
20	neo-menthyl acetate	1268	0.24
21	menthyl acetate	1287	3.23
22	iso-menthyl acetate	1302	0.16
23	β-bourbonene	1381	0.17
24	β-elemene	1386	0.11
25	β-caryophyllene	1416	1.74
26	aromadendrene	1438	0.31
27	α-humulene	1448	0.20
28	germacrene D	1477	1.51
29	δ-selinene	1492	0.30
30	spathulenol	1580	0.11
31	cubenol	1588	0.13
32	viridiflorol	1597	0.38
	Total		99.07

* RIs (Retention Indexes) calculated relative to C6–C28 n-alkanes on the HP- 5 column

Among the identified compounds, the dominant oil components were menthone (37.02%), menthol (29.57%) and isomenthone (9.06%), followed by other oxygenated monoterpenes [1,8-cineole (4.52%), menthyl acetate (3.23%), neo-menthol (2.52%) and piperitone (1.38%)], monoterpene hydrocarbons [sabinene (1.26%) and p-cymene (1.20%)] and sesquiterpene hydrocarbons [β-caryophyllene (1.74%) and germacrene D (1.51%)]. The components listed constituted 93.01% of total oil mass. Concentrations of all other components varied from 0.09 to 0.78%.

Comparing our results with literature data relating to peppermint composition, we found the EO originating from Serbia to be highly consistent with previous studies. Zeković et al. (2009) had found that the main components of an oil from peppermint collected in Vojvodina were menthone (37.15%), menthol (30.67%) and iso-menthone (10.33%). Similarly, Šarić et al. (2014) found the concentrations of menthol and menthone, as the main compounds of a peppermint essential oil from Vojvodina, were 38.82 % and 26.12 %, respectively. Soković et al. (2009) reported that in addition to menthol (37.4%) and menton (12.7%), menthyl acetate (17.4%) was also a dominant fraction of the Serbian mint oil. In addition, Samojlik et al. (2012) found the same compounds as dominant in Serbian (Vojvodina) peppermint oil [menthol (39.6%), menthyl acetate (10.4%) and menthone (8.9%)]. The similarity of results obtained in our study and several others confirms stable composition of *M. piperita* EOs from plants collected from no distant territory.

Growth of the isolates was inhibited by four tested EOs applied in a range of concentrations from 0.02-0.64 μl ml^{-1} (Table 1). Only two oils, yarrow and juniper, showed neither inhibitory nor lethal effects to the isolate tested. Growth inhibition of the tested pathogen after two days was achieved by oils from basil, peppermint, St. Johns wort and walnut. Lethal effect on the pathogen was exhibited only by peppermint oil.

The strongest growth inhibitor of *T. aggressivum* f. *europaeum* T77 was basil oil, having the MIC value of 0.02 μg ml^{-1}. The following oils also exhibited good inhibitory effects to isolate T77: peppermint, St. Johns wort and walnut, with respective MIC values of 0.04, 0.08, and 0.16 μg ml^{-1}. Yarrow oil showed the lowest MIC value of 0.64 μg ml^{-1}, while juniper oil did not inhibit pathogen growth at all. Fungicidal effect was shown only by peppermint oil, having MFC value of 0.64 μg ml^{-1} after four-day exposure.

Various *Mentha* species (*Lamiaceae*) have been recognized as plants with many useful pharmacological properties. They have been used for their flavours, in herbal medicine and as antimicrobial agents. It has been reported that EOs of peppermint (*M. piperita*) and spearmint (*M. spicata*) from Serbia possess great antifungal potential as they inhibited the growth of *T. viride* (MIC = 2.5 μl ml^{-1}) and three other pathogens of the button mushroom: *Lecanicillium fungicola,*

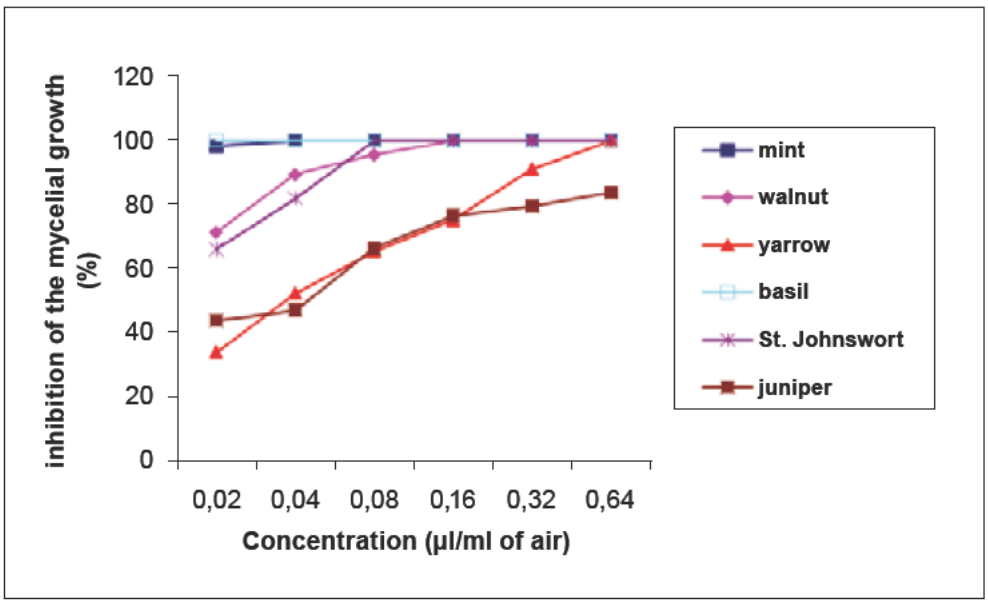

Figure 1. Effects of the volatile phase of essential oils on the growth of *Trichoderma aggressivum* f. *europaeum* T77 isolate *in vitro* after four-day exposure

T. harzianum and *P. tolaasii* (Soković & van Griensven, 2006; Soković et al., 2009). Bouchra et al. (2003) had found that *M. pulegium* exhibited moderate activity against *Botrytis cinerea*, and inhibition of mycelial growth of 58.5% at 250 µl ml^{-1}. We also found a remarkable activity of peppermint oil against *T. aggressivum*, having both fungicidal and inhibitory effects.

Sweet basil *Ocimum basilicum* L (*Lamiaceae*) is usually used in herbal medicine. A previous study in Iran had shown only low activity of sweet basil EO, with 42.5% inhibition of *Botrytis cinerea* mycelial growth at 0.5 µl ml^{-1} using the macrodilution method (Abdolahi et al., 2010). However, we found that basil oil from Serbia inhibited the growth of *T. aggressivum* 100% at 0.02 µl ml^{-1}.

The Serbian flora includes 19 species of *Hypericum* L., (*Hypeicaceae*) (Stjepanović-Veseličić, 1972). St. John's wort (*Hypericum perforatum* L.) is used as herbal medicine with antidepressant, antiviral and antibacterial effects. St. John's wort EO from Vranje-Barelić, Serbia has been found to have MIC of 25 µl ml^{-1} against *P. tolaasii*, a bacterial pathogen of *A. bisporus* (Saroglou et al., 2007). In our study, its EO from plants collected in Ljubovija showed higher activity against *T. aggressivum* (0.08 µl ml^{-1}).

Yarrow (*Achillea mylefollium* L., *Asteraceae*) is a native herb in Serbia and has been used as one of the principal constituents of traditional cures and remedies. It has been used in medicine because of its anti-inflammatory, spasmolytic, hemostatic and digestive effects. Interestingly, no antimicrobial activity of yarrow extracts or oils has been observed yet, although the EO of two other species of this genus, namely *A. clavennae* and *A. alpine*, respectively, have been recognized to have some antibacterial properties (Woods-Panzaru et al., 2009). In our study, only a weak inhibitory effect of yarrow oil to the pathogen was found (MIC value of 0.64 µl ml^{-1}).

Persian or common walnut, *Juglans regia* L. (*Juglandaceae*) is an important deciduous tree commercially cultivated in Serbia. Green walnuts, shells, kernels and seeds, bark and leaves have been used in pharmaceutical and cosmetic industries. Walnut leaves are considered a source of healthcare compounds and have been intensely used in traditional medicine for treatment of venous insufficiency and haemorrhoidal symptomatology and for its anti-diarrhetic and anti-helminthic properties. Antibacterial activities of leaf EO of walnut from India had been previously reported by Rather et al. (2012). We found a mild activity of walnut oil from Serbia against the pathogen (MIC=0.16 µl ml^{-1}).

The juniper (*Juniperus communis* L., *Cupressaceae*) is widespread in Serbia and it grows in temperate regions of Europe, Asia and North America. The EO of juniper female cones has diuretic and antiseptic properties and it is a gastrointestinal irritant. Examined by the diffusion method, a juniper oil from Croatia has shown strong fungicidal activity against yeast, yeast-like fungi and dermatophytes with MIC values below 10% (v/v) (Pepeljnjak et al., 2005). The juniper oil from Serbia only inhibited growth of the pathogen by 83.68% at 0.64 µl ml^{-1} (Figure 1).

Among the six essential oils analyzed, those of peppermint and basil demonstrated the strongest antifungal activity against *T. aggressivum* f. *europaeum*. Only peppermint oil exhibited lethal effect on the pathogen. The weakest inhibitory effect was displayed by yarrow oil. The oil of juniper had no antifungal effect at all. These results show that peppermint oil has a potential for further *in vivo* experiments against *T. aggressivum* f. *europaeum*.

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Antifungalna aktivnost šest etarskih ulja poreklom iz Srbije na *Trichoderma aggressivum* f. *europaeum*

REZIME

Ispitana je inhibitorna i fungicidna aktivnost šest etarskih ulja ekstrahovanih iz biljaka poreklom iz Srbije na *Trichoderma agressivum* f. *europaeum*. Patogen je prouzrokovač zelene plesni šampinjona, bolesti koja nanosi najviše štete u prinosu. Najveću aktivnost je ispoljilo ulje bosiljka (*Ocimum basilicum* L.) i pitome nane (*Mentha piperita* L.). Niža aktivnost je uočena kod ulja kantariona (*Hypericum perforatum* L.) i oraha [*Juglans regia* (F)]. Ulja ekstrahovana iz hajdučke trave (*Achillea millepholium* L.) i kleke (*Juniperus communis* L.) ispoljila su najmanju aktivnost. Ulje pitome nane je ispoljilo fungicidni efekat na patogena, sa minimalnom fungicidnom koncentracijom od 0.64 µl ml^{-1}. Najzastupljenije komponente etarskog ulja nane su bile menton (37.02%), mentol (29.57%) i izomenton (9.06%).

Ključne reči: Etarska ulja; Trichoderma; jestive gljive