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ANTIFUNGAL ACTIVITY OF *HELICHRYSUM ITALICUM* (ROTH) G. DON (ASTERACEAE) ESSENTIAL OIL AGAINST FUNGI ISOLATED FROM CULTURAL HERITAGE OBJECTS

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Abstract - There is considerable interest in the use of essential oils as alternative methods to control micromycetes from cultural heritage objects. We investigated the chemical composition and antifungal activity of the essential oil of *Helichrysum italicum*. The main components of the oil were γ-curcumene (22.45%), α-pinene (15.91 %) and neryl acetate (7.85 %). *H. italicum* essential oil showed moderate antifungal activity against fungi isolated from cultural heritage objects. The most susceptible fungi to oil treatment were *Epicoccum nigrum* and *Penicillium* sp., while the most resistant was *Trichoderma viride*. The *H. italicum* essential oil showed demelanizing activity against *Aspergillus niger*.

Key words: antifungal activity; demelanization; essential oils; *Helichrysum italicum* (Roth) G. Don

INTRODUCTION

The genus *Helichrysum* (family Asteraceae, tribe Inuleae) consists of approximately 600 species widespread all over the world and 25 species native to the Mediterranean area (Morone Fortunato et al., 2010). The name of the genus is derived from the Greek words "*helios*" (sun) and "*chryos*" (gold) and relates to the typical long-lasting bright yellow inflorescences, known as gold-everlasting or eternal flowers (Pignatti, 1982). The best investigated species of this genus is *Helichrysum italicum* (Roth) G. Don (*immortelle* on Italian), a woody dwarf shrub with yellow flowers growing on dry cliffs and sandy soil widespread along the coast and on the islands of the Adriatic Sea (Mastelić et al., 2008). *H. italicum* has been widely used in folk medicine. In the Greek-Roman system of medicine, *H. italicum* was used as an anti-inflammatory and anti-infective plant (Ballero and Maxia, 2006). Also, dried flowers of *H. italicum* had a great reputation in traditional medicine as a choleretic, diuretic and expectorant (Chinou et al., 1996, 1997). The biological activities of the many metabolites of *H. italicum*, especially volatile components of the essential oils, produced in the glandular hairs present on the leaves and flower heads of the plant (Morone Fortunato et al., 2010), have been confirmed in many recent studies. Previous studies reported many different activities: anti-inflammatory (Sala et al. 2002), antioxidant (Rosa et al., 2007), anti-allergic (Chinou et al., 1997), antibacterial (Nostro et al., 2001) and antiviral (Appendino et al., 2007).

The aim of this study was to estimate the antifungal potential of *H. italicum* essential oil against selected fungal species isolated from wooden and stone cultural heritage objects. Literature reports regarding the antifungal properties of *H. italicum* essential oil are scarce. However, Angioni et al. (2003) reported strong antifungal activity of *H. italicum* essential oil against *Globisporangium ultimum* (Trow) Uzuhashi, Tojo & Kakish, *Athelia rolfsii* (Curzi) C.C. Tu & Kimbr and moderate activity against *Phytophthora capsici* Leonian and *Zymoseptoria tritici* (Desm.) Quaedvl. & Crous. Mastelić et al. (2005) reported that *H. italicum* essential oil could inhibit growth of *Candida albicans* (C.P. Robin) Berkhout.

MATERIALS AND METHODS

Essential oil

The essential oil used in this study was a commercial sample *H. italicum* (Herba d.o.o, Belgrade, Serbia) analyzed by the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade.

Tested fungi

The fungal isolates used in this research were *Aspergillus niger* Tiegh (wood, w), *Aspergillus ochraceus* G. Wilh (w), *Bipolaris spicifera* (Bainier) Subram (stone, s), *Epicoccum nigrum* Link (s), *Penicillium* Link sp. (w) and *Trichoderma viride* Pers (w). The molds were deposited with the Mycotheca of the Department for Algology, Mycology and Lichenology, Institute of Botany, Faculty of Biology, University of Belgrade. Isolates were maintained on malt extract agar (MEA), potato dextrose agar (PDA), stored at 4˚C and subcultured once a month.

Gas chromatography (GC) and GC-mass spectrometry (GC/MS)

Qualitative and quantitative analyses of the EOs were performed using GC and GC-MS. The GC analysis of the oil was carried out on a GC HP-5890 II apparatus, equipped with a split-splitless injector, attached to a HP-5 column (25 m × 0.32 mm, 0.52-μm film thickness) and fitted to FID. Carrier gas flow rate (H2) was 1 ml/min, split ratio 1:30, injector temperature 250°C, detector temperature 300°C, while the column temperature was linearly programmed from 40-240°C (at the rate of 4°/min). The same analytical conditions were employed for GC-MS analysis, where a HP G 1800C Series II GCD system, equipped with a HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness) was used. The transfer line was heated to 260°C. The mass spectra were acquired in EI mode (70 eV), in m/z range 40-400. Identification of individual EO components was accomplished by comparison of retention times with standard substances and by matching mass spectral data with those held in the Wiley 275 library of mass spectra. Confirmation was performed using AMDIS software and literature (Adams, 2007). Area percentages obtained by FID were used as a base for the purpose of quantitative analysis.

Micro-atmosphere method

The following method allows study of the effect of the volatile fractions of the EO. The test was performed in sterile Petri plates (85 mm Ø) containing 20 ml of MEA (Maruzzella and Sicurella, 1960). After the inoculation of the tested fungi at the center of the MEA, the Petri plates were overturned. Sterilized filter paper disc was placed in the center of the Petri plate lid soaked with various amount of EO in final concentrations of 10, 25, 50, 75 and 100 μL mL^{-1}. The Petri plates were incubated at 28 ± 1˚C. The growth of the tested fungi was measured after 21 days and percent of inhibition was computed after comparison with the control. Fungistatic effect was expressed in terms of mycelia growth inhibition (%) and calculated by the formula of Pandey et al. (1982):

$$\text{Mycelial growth inhibition (\%)} = 100\ (dc - dt)/dc$$

where dc = average diameter of fungal colony in control and dt = average diameter of fungal colony in treatment.

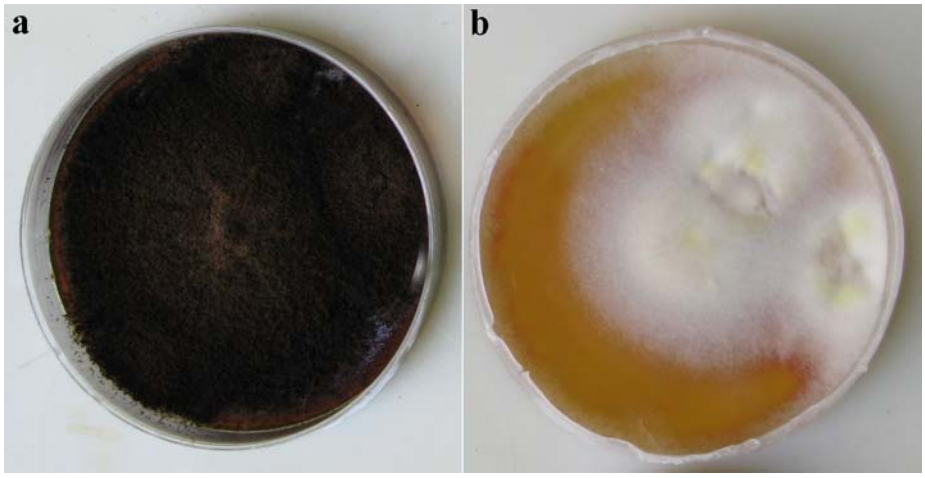

Fig. 1. Morpho-physiological changes documented in *Aspergillus niger* colony grown in microatmosphere conditions with *Helichrysum italicum* essential oil. **a.** Control; **b.** Albino colony grown at concentration of 100 μL mL^{-1}.

Statistical analyses

One-way ANOVA was performed for mycelia growth assay using Microsoft office Excel 2007. A P value less than 0.05 was considered statistically significant.

RESULTS

The *H. italicum* essential oil predominantly contained sesquiterpene hydrocarbons (60.66%), followed by monoterpene hydrocarbons (19.83%). The oxygenated monoterpenes comprised 4.49% and the sesquiterpenes class 3.73% of the total oil. Results of the essential oil analysis showed a total of 60 components (98.47% of oil) (Table 1). The main component of the oil was γ-curcumene (22.45%). Other components present in significant percentage were α-pinene (15.91%), neryl acetate (7.85%), and β-selinene (6.94%).

Fungi tested in the micro-atmosphere method showed different susceptibility to *H. italicum* essential oil. The most resistant species was *T. viride*. The mycelial growth of this fungus was not inhibited with any of the oil concentrations used in the experiment (Table 2). *E. nigrum* and *Penicillium* sp. were the most sensitive fungal species ($P<0.05$). For these fungi, inhibition of mycelial growth was documented at the concentration of 25 μL mL^{-1}. With an increase of *H. italicum* essential oil concentrations, a higher percentage of mycelial growth inhibition was recorded (Table 2).

In addition to inhibited growth, colonies of *A. niger* formed in the presence of *H. italicum* essential oil exhibited distinct morphological variations when compared to the control, such as visible loss of conidia melanization and a significantly lower number of conidial heads. Colonies with demelanized conidia were regarded as albino (Fig 1). After the reinoculation of albino colonies on sterile MEA, colonies of *A. niger* with typical species morphology were formed, suggesting that morphophysiological changes induced with oil were reversible. Demelanizing activity was documented toward *A. niger* only.

DISCUSSION

With regard to the chemical composition of *H. italicum* essential oils, Satta et al. (1999) suggested the presence of two different chemotypes, one rich in nerol and esters (chemotype A), and the other abundant with rosifoliol (chemotype B). However, Roussis et al. (2000) reported the presence of another, third, chemotype of *H. italicum* (chemotype C1) with essential oils rich in β-selinene, γ-curcumene and α-pinene. The oil examined in this research was dominated by γ-curcumene (22.45%) and a significant

Table 1. Chemical composition of *Helichrysum italicum* essential oil

Component	KIE[1]	KIL[2]	%
α-pinene	926.4	932	**15.91**
α-fenchene	939.2	945	0.39
camphene	949.1	946	0.20
β-pinene	969.0	969	0.31
p-cymene	1019.1	1020	0.06
limonene	1022.4	1024	2.52
1,8-cineole	1025.4	1026	0.30
isobutyl angelate	n/a*	1045	0.21
γ-terpinene	1052.9	1054	0.25
α-terpinolene	1082.3	1086	0.19
linalool	1096.5	1095	0.51
isoamyl 2-methyl butyrate	1100.1	1100	0.15
endo-fenchol	1108	1114	0.08
trans-pinocarveol	1133.3	1135	0.08
isoamyl tiglate	1149.6	1148	0.77
nerol oxide	1150.4	1154	0.11
borneol	1160.3	1165	0.10
cis-pinocamphone	1179.1	1172	0.53
terpinen-4-ol	1172.2	1174	0.55
α-terpineol	1185.8	1186	0.26
decanal	1201.8	1201	0.06
nerol	1224.7	1227	0.78
hexyl 2-methyl butanoate	1232.7	1233	0.16
hexyl 3-methyl-2-butenoate	1281.8	n/a*	0.30
2-undecanone	1291.4	1293	0.06
neryl acetate	1362.5	1359	**7.85**
α-ylangene	1364	1373	0.42
α-copaene	1368.7	1374	3.52
italicene	1395.8	1405	5.42
cis-α-bergamotene	1408.1	1411	1.44
trans-caryophyllene	1411.6	1417	4.74
trans-α-bergamotene	1428.6	1432	3.24
neryl propanoate	1449.4	1452	1.39
allo-aromadendrene	1464.4	1458	0.28
α-acoradiene	1471.0	1464	0.12
β-acoradiene	1473.8	1469	0.64
selina-4,11-diene	1468.2	1475	1.13
γ-curcumene	1474.3	1481	**22.45**
ar-curcumene	1477.0	1479	1.90
β-selinene	1479.1	1489	**6.94**
α-selinene	1488.1	1498	4.78
α-muurolene	1493.2	1500	1.39
β-curcumene	1505.0	1514	0.60
δ-cadinene	1516.0	1522	1.52
italicene ether	1526.6	1536	0.54
α-calacorene	1535.4	1544	0.13
trans-nerolidol	1557.1	1561	0.06
caryolan-8-ol	1561.5	1571	0.05
geranyl 2-methylbutyrate	1570.2	1574	0.57
neryl isovalerate	1573.7	1582	0.47
caryophyllene oxide	1585.5	1582	0.08

Table 1. *Continued*

Component	KIE[1]	KIL[2]	%
globulol	1589.8	1590	0.17
viridiflorol	1595.8	1592	0.40
rosifoliol	1599.1	1600	0.39
humulane-1,6-dien-3-ol	1604.0	1619	0.14
γ-eudesmol	1623.0	1630	0.17
β-eudesmol	1625.8	1649	0.06
selin-11-en-4-a-ol	1646.3	1658	0.40
epi-β-bisabolol	1661.7	1670	0.09
α-bisabolol	1663.2	1674	0.14
Grouped constituents			
Monoterpene hydrocarbons			19.83
Oxygenated monoterpenes			4.49
Sesquiterpene hydrocarbons			60.66
Oxygenated sesquiterpenes			3.73
Others			9.76
Total:			**98.47**

[1] Kovats retention index, experimental data
[2] Kovats retention index (Adams, 2007)
*not available

Table 2. Antifungal activity of *Helichrysum italicum* essential oil against selected fungi

Oil concentration (μL mL^{-1})	Mycelial growth inhibition (mean ± SE)*(%)					
	A.n	*A.o*	*B.s*	*E.n*	*P.*	*T.v*
10	0	0	0	0	0	0
25	0	0	0	24.31±2.16	7.26±1.40	0
50	0	0	0	59.66±2.03	12.50±1.66	0
75	2.67±1.4	9.1±0.81	18.67±1.33	65.33±3.72	31.68±8.23	0
100	29±1.4	11.5±1.22	42.33±6.17	76.93±4.05	59.56±4.07	0

*mean of three replication ($P < 0.05$); *A.n - Aspergillus niger*; *A.o - Aspergillus ochraceus; B.s - Bipolaris spicifera*; *E.n - Epicoccum nigrum*; *P. – Penicillium* sp. *T.v - Trichoderma viride*.

presence of α-pinene (15.91 %) and β-selinene (6.94 %), which places this oil in the chemotype group C1. The ability of *H. italicum* essential oil to inhibit mycelial growth of tested fungi was monitored using the micro-atmosphere method that allows estimation of the growth inhibition of mycelia exposed to oil vapor components. According to Angioni et al. (2003), the antimicrobial activity of *H. italicum* essential oils can be considered as moderate. Chinou et al. (1996) suggested that the antimicrobial activity of *H. italicum* oil was due to its richness in nerol and ester components (chemotype A). However, Roussis et al. (2000) pointed out that the essential oil of *H. italicum* synthesized during anthesis and rich in β-selinene, α-pinene and γ-curcumene (chemotype C1) have strong antibacterial activity. The tested fungi showed different susceptibility to oil treatment. *T. viride* appeared to be the most resistant, while *E. nigrum* and *Penicillium* sp. were the most sensitive. The resistance of *T. viride* can be explained by a variety of enzymes produced and secreted by mycelia that can detoxify oil components into inactive forms (Farooq et al., 2002). Although 100% of mycelia growth inhibition was not accomplished, even with the highest concentration of oil used in the experiment (100 μL mL^{-1}), the morpho-physiological variations documented in the *A. niger*

colonies suggested that the oil components interfered with fungal metabolism. It can be concluded that *H. italicum* essential oil can prevent *A. niger* from completing its life cycle, which was demonstrated with the depigmentation and scarce sporulation, leading to the formation of albino colonies. Conidia of some *Aspergillus* and *Penicillium* species contain pigments belonging to melanins: a green-colored chromoprotein and a black insoluble pigment (Eismann and Casadevall, 2012). Abundant sporulation of these fungi causes the formation of colonies in different shades of yellow, green, ochre, blue and black, etc. Alterations of *A. niger* colonies induced by *H. italicum* essential oil may be related to the interference of the oil components in melanin biosynthesis. Other essential oils can display demelanizing activity against different fungi. Sharma and Tripathi (2008) reported the visible pigmentation loss of *A. niger* colonies grown with essential oil isolated from *Citrus sinensis* (L.) epicarp. Conidia of different *Aspergillus* species (*A. flavus* Link, *A. parasiticus* Speare, *A. ochraceus*, *A. fumigatus* Fresenius and *A. niger*) lost their pigmentation when treated with *Hyptis suaveolens* (L.) Poit essential oil (Pessoa Moreira et al., 2010). Although there are reports that *B. spicifera* poroconidia can be demelanized when treated with *Nepeta rtanjensis* Diklić & Milojević essential oil (Ljaljević Grbić et al., 2011), the tested essential oil of *H. italicum* did not display any such activity, suggesting different target mechanisms of these oils. However, melanin production by certain fungi contributes to the virulence of human, animal and plant pathogenic fungi (Butler et al., 2001), and therefore the demelanization effect caused by interaction with essential oils, as antifungal agents, is significant.

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