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Full Length Research Paper

A detailed chemical composition and antimicrobial activity of *Hypericum richeri* Vill. subsp. *grisebachii* (Boiss.) Nyman essential oil from Serbia

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The essential oil of fresh aerial parts of *Hypericum richeri* subsp. *grisebachii* obtained by hydrodistillation was analyzed by gas chromatography (GC) and gas chromatography/mass spectral GC/MS. One hundred and five constituents identified accounted for 96.1% of the total oil. The major components of the oil were: germacrene D (13.1%), dodecanal (11.9%), β-caryophyllene (7.4%), β-pinene (5.8%), caryophyllene oxide (4.2%) and (*E*)-β-ocimene (3.3%). The volatile profile of *H. richeri* subsp. *grisebachii* was characterized by a large amount of sesquiterpenoids (56.2%), especially by hydrocarbons of this fraction (34.1%). The *in vitro* antimicrobial activity of essential oil was also examined against a panel of microbial strains by broth microdilution assay and it was found to have moderate effect against all tested microorganisms.

Key words: *Hypericum richeri* subsp. *grisebachii*, essential oil composition, antimicrobial activity, germacrene D, dodecanal.

INTRODUCTION

Hypericum L. (Guttiferae/Hypericaceae) is a genus represented by ca. 400 species, widespread in warm-temperate areas throughout the world, as well as on the Balkan Peninsula (Robson and Strid, 1986). Among them *Hypericum perforatum* is one of the best chemically investigated plant species. Recently, several studies have been published on essential oils of *Hypericum* species, including *Hypericum richeri* Vill., showing a considerable variation in chemical composition (Smelcerovic et al., 2007; Ferretti et al., 2005; Maggi et al., 2010). However, these studies did not discuss the chemical variability of *H. richeri* on the species and subspecies level. Therefore, this paper dills with the chemical composition of *H. richeri* subsp. *grisebachii* essential oil and its relationship with the same species already examined, from different collection sites. Plants of the genus *Hypericum* have found numerous ethnopharmacological uses (Menković et al., 2006). The extracts, as well as the oils of *Hypericum* species have been shown to possess significant antiviral, wound healing, antioxidant and antimicrobial activities (Rocha et al., 1995; Gudzic et al., 2002; Saroglou et al., 2007; Bilia et al., 2002; Cakir et al., 2004, 2005; Couladis et al., 2002). Some recent investigations have shown that *H. richeri* also possess a significant potential in this respect. *H. richeri* ethanol extract produces significant dose-dependent anti-inflammatory effect (Šavikin et al., 2007). In addition, Zdunić et al. (2010) results approve the usage of *H. richeri* oil extracts as an anti-inflammatory and gastroprotective agent.

Up to now, the antimicrobial activity of the *H. richeri* essential oil was the subject of two studies only (Maggi et al., 2010; Saroglou et al., 2007). Both studies found

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significant antimicrobial potential of the mentioned species. Thus, the objective of this study was also to determine antimicrobial activity of *H. richeri* subsp. *grisebachii* essential oil, in order to evaluate its potential for some pharmaceutical applications. As far as we know, we have reported herein minimal bactericidal and minimal fungicidal concentrations of *H. richeri* subsp. *grisebachii* essential oil for the first time.

MATERIALS AND METHODS

Plant material

Aerial parts of *H. richeri* subsp. *grisebachii* in the flowering phase were collected in the region of central Serbia (Željin Mountain) in July 2010. Voucher specimens were deposited in the Herbarium of Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU) under the acquisition number 16481.

Essential oil isolation

Fresh aerial parts (300 g) of *H. richeri* subsp. *grisebachii* were subjected to hydrodistillation for 2.5 h using an original Clevenger-type apparatus and yielded 0.03% (w/w) of pale yellow essential oil. The obtained oil was separated, dried over anhydrous magnesium sulfate and immediately analyzed.

Essential oil analyses

Chemical composition of the oil was investigated by GC and GC/MS. The GC/MS analyses (three repetitions) were carried out using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column HP-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 300°C, respectively. Oven temperature was raised from 70 to 290°C at a heating rate of 5°C/min and then isothermally held for 10 min. As a carrier gas helium at 1.0 ml/min was used. The sample, 1 µl of oil solution in diethyl ether (1: 100) was injected in a pulsed split mode (the flow was 1.5 ml/min for the first 0.5 min and then set to 1.0 ml/min throughout the remainder of the analysis; split ratio 40: 1). MS (electron impact) conditions were as follows: ionization voltage of 70 eV, acquisition mass range 35 to 500, scan time 0.32 s. Oil constituents were identified by comparison of their linear retention indices (relative to n-alkanes (Van den Dool and Kratz, 1963) on the HP-5MS column) with literature values (Adams, 2007) and their mass spectra with those of authentic standards, as well as those from Wiley 6, NIST02, MassFinder 2.3, and a homemade MS library with the spectra corresponding to pure substances and components of known essential oils, and wherever possible, by co-injection with an authentic sample.

GC (FID) analysis was carried out under the same experimental conditions using the same column as described for the GC/MS. The percentage composition of the oil was computed from the GC peak areas without any corrections.

Antimicrobial activity

The *in vitro* antimicrobial activity of *H. richeri* subsp. *grisebachii* essential oil was tested against a panel of strains belonging to the American Type Culture Collection Maryland, USA and National Collection of Type Cultures. Antibacterial activity was evaluated against two gram-positive and three gram-negative bacteria. Gram-positive bacteria used were *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 while gram-negative bacteria were *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NCTC 6017. The antifungal activity was tested against two organisms *Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 10231. The minimal inhibitory concentration (MIC) of *H. richeri* subsp. *grisebachii* essential oil was determined using a broth microdilution method in 96-well microtitre plates reported by Sarker et al. (2007). To determine minimal bactericidal concentration/ minimal fungicidal concentration (MBC/ MFC), broth was taken from each well without visible growth and inoculated on Mueller Hinton agar (MHA) for 24 h at 37°C for bacteria or on Sabouraud dextrose agar (SDA) for 48 h at 28°C for fungi. Doxycycline and nystatin served as positive controls, while the solvent (10% aqueous DMSO) was used as a negative control. One non-inoculated well, free of antimicrobial agent was also included to ensure medium sterility. Tests were carried out in triplicate.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The compounds identified in *H. richeri* subsp. *grisebachii* essential oil are listed in Table 1. One hundred and five constituents accounted for 96.1% of the total oil. Germacrene D (13.1%), dodecanal (11.9%), β-caryophyllene (7.4%), β-pinene (5.8%), caryophyllene oxide (4.2%) and (*E*)-β-ocimene (3.3%) were the most abundant components. The oil was characterized by a large amount of terpenoids (76.1%) unevenly distributed between mono- and sesquiterpenoids (Table 1). Sesquitrepenoids were qualitatively and quantitatively the most abundant fraction (with 52 compounds amounting 56.2%). The ratio of hydrocarbons (34.1%) and oxygenated derivatives of this fraction (22.1%) was relatively similar (Table 1) while monoterpenoid fraction was dominated by hydrocarbons (17.1%). The oxygenated monoterpenes amounted to only 2.8%. Non-terpenoids participated almost as much as monoterpenoids in percentage oil composition (18.8 and 19.9%, respectively), making up about one fifth (1/5) of the total oil (Table 1). It is interesting to mention that fatty acid and fatty acid derivatives (FAD) took percentage prevalence among non-terpenoids, being presented with 14.2% (Table 1), due to the second most abundant compound of the examined oil, dodecanal (11.9%). If we compare our results with recently published data (Smelcerovic et al., 2007; Ferretti et al., 2005; Saroglou et al., 2007; Maggi et al., 2010), it is worthy of comment both qualitative and quantitative differences in chemical composition, not only on the species, but also on the subspecies level. Smelcerovic et al. (2007) reported (*E*)-anethole (9.5%) as a major component of *H. richeri* essential oil, followed by

Table 1. Percentage composition of *Hypericum richeri* subsp. *grisebachii* essential oil.

RI§	Component	Percentage	Class	Identification
900	Nonane	0.4	An	a, b, c
928	α-Thujene	0.1	M	a, b
937	α-Pinene	2.8	M	a, b, c
953	Camphene	tr	M	a, b, c
957	Thuja-2,4(10)-diene	tr	M	a, b
976	Sabinene	0.1	M	a, b
982	β-Pinene	5.8	M	a, b, c
991	Myrcene	1.5	M	a, b, c
991	2-Pentylfuran	tr	FAD	a, b
1008	α-Phellandrene	0.3	M	a, b
1019	α-Terpinene	0.1	M	a, b
1027	*p*-Cymene	tr	M	a, b, c
1031	Limonene	0.6	M	a, b, c
1036	(*Z*)-β-Ocimene	3.3	M	a, b
1047	(*E*)-β-Ocimene	1.9	M	a, b
1060	γ-Terpinene	0.4	M	a, b, c
1092	Terpinolene	0.2	M	a, b
1100	Undecane	2.2	An	a, b, c
1118	Fenchol*	0.2	MO	a, b
1130	α-Campholenal	0.3	MO	a, b
1144	*trans*-Pinocarveol	0.2	MO	a, b
1148	*trans*-Verbenol	tr	MO	a, b
1154	Camphene hydrate	tr	MO	a, b
1167	Pinocarvone	0.1	MO	a, b
1175	*p*-Mentha-1,5-dien-8-ol	0.1	MO	a, b
1181	Terpinen-4-ol	0.3	MO	a, b
1194	α-Terpineol	0.8	MO	a, b
1200	cis-Dihydro carvone	0.4	MO	a, b
1201	*trans*-Dihydro carvone	0.4	MO	a, b
1206	Decanal	1.3	FAD	a, b
1236	(*Z*)-3-Hexenyl-3-methylbutanoate	0.3	FAD	a, b
1300	Tridecane	0.1	An	a, b, c
1307	Undecanal	0.4	FAD	a, b
1353	α-Cubebene	0.1	S	a, b
1376	α-Ylangene	0.1	S	a, b
1380	α-Copaene	0.5	S	a, b
1380	1-Undecanol	0.6	O	a, b
1390	β-Bourbonene	tr	S	a, b
1390	β-Cubebene	tr	S	a, b
1396	β-Elemene	1	S	a, b
1409	Dodecanal	11.9	FAD	a, b
1424	β-Caryophyllene	7.4	S	a, b, c
1435	β-Copaene	0.3	S	a, b
1456	*trans*-Muurola-3,5-diene	0.1	S	a, b
1458	(*E*)-β-Farnesene	0.6	S	a, b
1459	α-Humulene	1.1	S	a, b, c
1467	*allo*-Aromadendrene	0.2	S	a, b
1465	*cis*-Muurola-4(14),5-diene	0.3	S	a, b

Table 1. Contd.

1473	*trans*-Cadina-1(6),4-diene	0.2	S	a, b
1481	γ-Muurolene	0.6	S	a, b
1482	α-Amorphene	tr	S	a, b
1487	Germacrene D	13.1	S	a, b
1492	β-Selinene	1.1	S	a, b
1494	*trans*-Muurola-4(14),5-diene	tr	S	a, b
1502	Bicyclogermacrene	1.3	S	a, b
1502	α-Muurolene	0.7	S	a, b
1509	(*E*,*E*)-α-Farnesene	0.6	S	a, b
1510	Germacrene A	0.9	S	a, b
1519	γ-Cadinene	0.7	S	a, b
1527	δ-Cadinene	2.4	S	a, b
1530	Zonarene	0.3	S	a, b
1530	Methyl dodecanoate	0.1	FAD	a, b
1537	*trans*-Cadina-1,4-diene	0.2	S	a, b
1542	α-Cadinene	0.2	S	a, b
1548	α-Calacorene	0.1	S	a, b
1560	Salviadienol	0.4	SO	a, b
1567	*trans*-Nerolidol	0.7	SO	a, b
1570	1,5-epoxysalvial-4(14)-ene*	0.5	SO	a, b
1584	Spathulenol	2.1	SO	a, b
1590	Caryophyllene oxide	4.2	SO	a, b
1596	Globulol	0.8	SO	a, b
1597	salvial-4(14)-en-1-one	0.6	SO	a, b
1598	Viridiflorol	0.3	SO	a, b
1608	Rosifoliol	tr	SO	a, b
1609	Ledol	0.6	SO	a, b
1620	Humulene epoxide II	1	SO	a, b
1621	1,10-di-*epi*-Cubenol	tr	SO	a, b
1626	Junenol	0.5	SO	a, b
1634	1-*epi*-Cubenol	0.8	SO	a, b
1634	Acorenol*	0.6	SO	a, b
1646	*epi*-α-Cadinol (syn. τ-cadinol)	tr	SO	a, b
1647	*epi*-α-Murrolol (syn. τ-muurolol)	3	SO	a, b
1651	α-Muurolol (syn. torreyol)	tr	SO	a, b
1655	*cis*-Guaia-3,9-dien-11-ol	tr	SO	a, b
1660	α-Cadinol	2.5	SO	a, b
1661	Selin-11-en-4-ol*	1.8	SO	a, b
1692	Germacra-4(15),5,10(14)-trien-1-α-ol	0.4	SO	a, b
1697	Acorenone*	1.3	SO	a, b
1700	Heptadecane	tr	An	a, b, c
1845	Hexahydrofarnesyl acetone	0.3	CR	a, b
1900	Nonadecane	0.1	An	a, b, c
1950	Isophytol	0.1	DO	a, b
2000	Eicosane	tr	An	a, b, c
2037	Octadecanal	tr	FAD	a, b
2100	Heneicosane	tr	An	a, b, c
2114	Phytol	1.2	DO	a, b
2197	1-Docosene	tr	O	a, b

Table 1. Contd.

2300	Tricosane	0.2	An	a, b, c
2400	Tetracosane	tr	An	a, b, c
2500	Pentacosane	0.2	An	a, b, c
2641	Tetracosanal	0.1	FAD	a, b
2700	Heptacosane	0.1	An	a, b, c
2845	Hexacosanal	0.1	FAD	a, b
2900	Nonacosane	0.4	An	a, b, c
3100	Hentriacontane	tr	An	a, b, c
	Total	96.2		
	Monoterpenoids	19.9		
	Hydrocarbons	17.1		
	Oxygenated	2.8		
	Sesquiterpenoids	56.2		
	Hydrocarbons - M	34.1		
	Oxygenated - MO	22.1		
	Diterpenoids - DO	1.3		
	Non-terpenoids	18.8		
	n-Alkanes - A_n	3.7		
	Carotenoid derived compounds - CR	0.3		
	Fatty acids and fatty acid derivatives - FAD	14.2		
	Others - O	0.6		

§Compounds listed in order of elution on HP-5MS column. RI: experimentally determined retention indices on the mentioned column by co-injection of a homologous series of n-alkanes; tr: less than 0.05%; syn: synonym; *correct stereoisomer not determined; a: comparison of experimentally determined RI with those given in literature; b: identification based on comparison of mass spectra and c: identity confirmed by co- injection of an authentic sample.

by globulol (9.4%), n-nonane (7.9%), caryophyllene oxide (7.1%) and hexadecanoic acid (6.2%), differing significantly from the composition under study (with complete lack of the (*E*)-anethole in our essential oil composition). Additionally, the oil com-position of the same species (from Italy) was the aim of the study conducted by Ferretti et al. (2005), who declared quite diverse chemical profile from ours, with (*Z*)-β-ocimene (19.5%), n-nonane (13.8%), β-bisabolene (8.7%) and (*E*)-β-ocimene (8.0%) as the most abundant compounds. We would like to point out that the species from the afore-mentioned studies (Smelcerovic et al., 2007; Ferretty et al., 2005) were not determined on the subspecies level (subsp. *richeri* or subsp. *grisebachii*, assumed subspecies for the regions in question).

Essential oil composition of the other sample from central Italy (this time with specified subspecies), *H. richeri* subsp. *richeri*, has been published by Maggi et al. (2010). They reported germacrene D (26.9%), (*Z*)-β-ocimene (11.2%), (*E*)-β-ocimene (5.7%) and α-cadinol (5.5%) as the major compounds (Maggi et al., 2010). Volatile profile of this sample was similar with the one presented here, regarding the main compound-germacrene D, but also different taking into account other abundant components. It is interesting to note that in the oil compositions of both Italian samples as well as the sample reported by Smelcerovic et al. (2007), dodecanal the second most abundant compound of essential oil composition under study was completely absent. Not long ago, Saroglou et al. (2007) published essential oil composition of *H. alpinum* Waldst. and Kit. from Serbia with the following major components: β-pinene (13.3%), γ-terpinene (7.7%), (*E*)-caryophyllene (6.5%) and caryophyllene oxide (4.8%), being comparable and similar with our sample. It should be noted here that classification and determination of *Hypericum* species in Flora of Serbia (Stjepanović-Veselič́ić, 1972) brought additional confusion. Namely, Flora of Serbia (Stjepanović-Veselič́ić, 1972) recognized *H. alpinum* Waldst. and Kit., *H. transsilvanicum* Čelak. and *H. richeri* Vill. as three

Table 2. Minimal inhibitory (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) of *Hypericum richeri* subsp. *grisebachii* essential oil.

Micoorganism		Tested sample (mg/ml)		Doxycycline (µg/ml)		Nystatin (µg/ml)	
		MIC	MBC/MFC	MIC	MBC	MIC	MFC
Gram-positive	*S. aureus*	1.625	6.50	0.780	6.250	NT	NT
	B. subtilis	6.50	25.00	1.560	1.560	NT	NT
Gram-negative	*P. aeruginosa*	6.50	12.00	12.50	12.50	NT	NT
	S. abony	6.50	12.00	6.250	6.250	NT	NT
	E. coli	6.50	25.00	0.780	0.780	NT	NT
Yeast	*C. albicans*	1.625	6.50	NT	NT	6.250	6.250
Fungus	*A. niger*	6.50	50.00	NT	NT	0.780	0.780

NT: not tested.

different species while Flora Europaea (Robson, 1968) considers *H. alpinum* Waldst. and Kit. and *H. transsilvanicum* Čelak. as synonyms of the species *H. richeri* Vill. subsp. *grisebachii* (Boiss.) Nyman.

Our investigation of *H. richeri* subsp. *grisebachii* essential oil chemical composition (and its comparison with composition of aforementioned related species) rather corroborates classification of *Hypericum* species described in Flora Europaea (1968) than that in Flora of Serbia (1968).

Antimicrobial activity

The results of antimicrobial activity of the *H. richeri* subsp. *grisebachii* essential oil against five bacterial and two fungal strains are listed in Table 2. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration/ minimal fungicidal concentration (MBC/ MFC) determinations were obtained by broth micro-dilution assay. In general, the oil showed moderate activity against all tested microorganisms. The results indicated that MIC values against tested organisms ranged from 1.625 to 6.50 mg/ml while MBC/MFC values ranged from 6.50 to 50 mg/ml. Among bacteria, *H. richeri* subsp. *grisebachii* essential oil was the most effective against gram-positive bacteria *S. aureus* (MIC = 1.625 mg/ml, MBC = 6.50 mg/ml) (Table 2). As for the antibacterial effect, the oil showed antifungal activity against both tested fungal strains, and was found to be more effective against the pathogenic yeast *C. albicans* with the same activity as for the aforementioned bacteria (MIC = 1.625 mg/ml, MFC = 6.50 mg/ml). The observed microbistatic and fungistatic concentrations with value of 1.625 mg/ml (for *S. aureus* and *C. albicans*) were four times lower in comparison with MIC values for the other tested strains (MIC amounted for 6.50 mg/ml). The assayed sample was less effective than the antibiotic/ antimycotic used as a referent standard (Table 2). Up to now, the antimicrobial activity of *H. richeri* essential oil and related subspecies (*richeri* and *grisebachii*) was the subject of two studies only (Saroglou et al., 2007; Maggi et al., 2010). Saroglou et al. (2007) reported MIC values for the set of strains that differed significantly from the set of our microorganisms. Their sample showed much better antibacterial activity against *S. aureus* and *E. coli* (12.50 and 50 µg/ml, respectively) than those obtained in this work (Saroglou et al., 2007). It is also evident the lack of the antimicrobial activity against *P. aeruginosa* and *C. albicans* in the study of Saroglou et al. (2007) while our results showed effect on these strains, being best against *S. aureus* and *C. albicans*. *H. richeri* subsp. *richeri* essential oil examined by Maggi et al. (2010) was the most active against *B. subtilis*, *S. aureus* and *C. albicans* (78, 155 and 155 µg/ml, respectively); the last two strains were also the most susceptible to the action of the sample examined in this paper (although it was found to be less effective at higher MIC values).

The antimicrobial potential of *H. richeri* subsp. *grise bachii* essential oil could be explained by its high content of terpenoids. This activity is suspected to be associated with the high percentage of sesquiterpenoid fraction, since it was previously reported that β-caryophyllene, β-pinene and caryophyllene oxide possessed moderate to strong activities against a number of microorganisms (Magiatis et al., 2002; Bougatsos et al., 2004). A synergistic effect of the oil compounds should also be taken into account in damaging pathogen cells. The main conclusion from the earlier stated data is that *H. richeri* subsp. *grisebachii* essential oil, in comparison with other oil samples belonging to the same species, showed considerable variation in chemical composition and it was

found to be the most related with *H. alpinum* essential oil. The results of antimicrobial screening demonstrated moderate activity against all tested microorganisms. Opposite to the chemical composition, better correlation was noticed in antimicrobial activity (concerning the effect against the same microorganisms) between oil samples belonging to plant species different on subspecies level (subsp. *richeri* - by Maggi et al. (2010) and subsp. *grisebachii* - sample in this study) than the samples from the same region (Saroglou et al., 2007 and present work).

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