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Chemical composition and antifungal activity of essential oil of *Salvia sclarea* L. from Bulgaria against clinical isolates of *Candida* species

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ABSTRACT

The essential oil of *Salvia sclarea* L., growing in Bulgaria, was analyzed by gas chromatography – mass spectrometry. A total of 52 different compounds were identified, representing 98.25% of total oil content. Linalyl acetate (56.88%) and linalool (20.75%) were determined as major essential oil constituents, followed by germacrene D (5.08%) and β-cariophyllene (3.41%). Antifungal activities of clary sage essential oil and major compounds linalyl acetate and linalool against 30 clinical isolates, belonging to species *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata* and *Candida parapsilosis* were evaluated. Essential oil characterized with stronger anticandidial activity in comparison with pure compounds.

Key words: antifungal activity, *Candida*, clinical isolates, essential oil, *Salvia sclarea*

Introduction

Genus *Salvia* is the largest in *Lamiaceae* family comprises about 700-900 species (Bown, 1995; Chevallier, 1996; Kintzios, 2000). Among these species *Salvia sclarea* L. is one of the most important from industrial point of view, because of its application as flavouring agent and essential oil bearing plant (Georgiev & Stoyanova, 2006; Dikova, 2009). Clary sage is widely distributed in northern Mediterranean, Central Asia and some areas in North Africa (Bown, 1995; Carrubba et al., 2002). The essential oil from *S. sclarea* has been found to demonstrate antibacterial, antifungal, antiinflamatory, antioxidant, antiviral and insecticidal (Peana et al., 1999; Pitarokili et al., 2002; Gülçin, 2004; Fraternale et al., 2005; Georgiev & Stoyanova, 2006; Jirovetz et al., 2006; 2007; Kuźma et al., 2009) properties and have been used traditionally for medicinal purposes. Gas chromatographic and mass spectrometric analysis of commercial clary sage essential oils from Russia, Bulgaria, USA, China, France and Tajikistan led to identification of linalool and linalyl acetate as major constituents of the oil (Pitarokili et al., 2002; Cai et al., 2006; Jirovetz et al., 2006, 2007; Sharopov & Setzer, 2012). Notable differences in the linalool/linalyl acetate ratio among oil samples from different geographic reagions were detected. Other chemotypes of *S. sclarea* essential oil from Israel, Italy and Tunisia were also published (Elnir et al., 1991; Carrubba et al., 2002; Fraternale et al., 2005).

Due to the constantly increasing number of fungal diseases caused by resistant strains of *Candida* species and insufficient effectiveness of traditionally applied azole preparations, the search of new and effective natural antifungals dramatically increased (Pauli, 2006).

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The aim of the present study was to determine the chemical composition and antifungal activity of essential oil from *S. sclarea* L. from Bulgaria, against thirty clinical isolates of different *Candida* species.

Materials and Methods

Samples

Essential oil of *S. sclarea* L. is commercial sample purchased from Vigalex Ltd. (Gurkovo, Bulgaria). Pure linalool and linalyl acetate were purchased form Sigma-Aldrich Co. (Vienna, Austria).

GC analysis

GC/FID and GC/MS analyses were carried out simultaneously using a Finnigan ThermoQuest TraceGC with a dual split/splitless injector, a FID detector and a Finnigan Automass quadrupole mass spectrometer. One inlet was connected to a 50 m x 0.25 mm x 1.0 μm SE-54 (5% Diphenyl, 1% vinyl-, 95% dimethyl-polysiloxane) fused silica column (CS Chromatographie Service, Germany), the other injector was coupled to a 60 m x 0.25 mm x 0.25 μm Carbowax 20M (polyethylene glycol) column (J & W Scientific, USA). The two columns were connected at the outlet with a quartz Y connector and the combined effluents of the columns were split simultaneously to the FID and MS detectors with a short (ca. 50 cm) 0.1 mm ID fused silica restrictor column as a GC/MS interface. The carrier gas was helium 5.0 with a constant flow rate of 1.5 mL/min., injector temperature was 230°C, FID detector temperature 250°C, GC/MS interface heating 250°C, ion source at 150°C, EI mode at 70 eV, scan range 40 - 300 amu. The following temperature program was used: 46°C for 1 min to 100°C at a rate of 5°C/min.; 100°C to 230°C at 2°C/min; 230°C for 13.2 min. Identification was achieved using Finnigan XCalibur 1.2 software with MS correlations through the NIST (2008), Adams essential oils (Adams, 2007), MassFinder and our own library. Retention indices of reference compounds and from literature data (Jennings & Shibamoto, 1980; Kondjoyan & Berdarqué, 1996; Joulian & König, 1998) were used to confirm peak data. Quantification was achieved through peak area calculations of the FID chromatogram.

Test microorganisms

To evaluate the antimicrobial activity of *S. sclarea* L. essential oil, fourteen strains of *C.albicans* (thirteen clinical isolates and one reference strain ATCC 10231), four strain of *C. parapsilosis*, four strains of *C. krusei*, four strains of *C. glabrata* and four strains of *C. tropicalis* were used. The strains were obtained from National Reference Laboratory of Mycology at National Center of Infectious and Prasitic Diseases, Sofia, Bulgaria and Department „Biochemistry and microbiology" at University of Plovdiv. The strains were maintained on Sabouraud Dextrose Agar with Chloramphenicol (SDA, HiMedia).

Antimicrobial testing

Antimicrobial testing of essential oil was performed according to CLSI M27-A3 reference Serial Broth Microdilution Method (2008). A stock solution was prepared by diluting the essential oil sample in DMSO (Sigma-Aldrcih, Co). Serial two-fold dilutions of the stock solution were prepared in RPMI-1640 broth medium buffered to pH 7.0 with 0.165 mol/l MOPS buffer (3-N-porpholinopropanesulfonic acid, Sigma-Aldrcih,Co) to reach final concentrations of the oil ranging from 2048 μg/ml to 1 μg/ml and were distributed in 96 wells microtitration plates. The final concentration of DMSO did not exceed 1% and did not influence the growth of yeasts. Control samples of inoculated broth medium with and without solvent were also incubated under the same conditions. Each well was inoculated with 0.1 cm^{-3} inoculum suspension ($0.5x10^3$-$2.5x10^3$ cfu/ml), prepared according to CLSI M27-A2 (2008). After 48 h incubation at 35°C, microbial growth was evaluated visually and the Minimal Inhibitory Concentration (MIC) was determined. MIC was defined as the lowest concentration at which total inhibition of microbial growth was detected. MIC was presented as an average value of the MICs detected for the separate strains within the species. MICs of Fluconazole (FLC), Itraconazole (IT) and Ketoconazole (KT) were also determined by HiComb™ MIC Test (HiMedia), according to manufacturer's intructions. To determine Minimal Fungicidal Concentration (MFC) of the essential oil 0.1 ml of each dilution showing no growth was spread on PDA. The inoculated Petri dishes were incubated at 35°C for 48 h. The colony forming units were counted and compared with control dishes. MFC was defined as the lowest concentration that killed more than 99.9% of the initial inoculum. MFC was presented as an average value of the MFCs detected for the separate strains within the species.

Results and Discussion

Using GC/FID and GC/MS chemical composition of essential oil from *S. sclarea* L., growing in Bulgaria was analyzed. The results obtained are shown in Table 1. Fifty

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two constituents were identified by the first column (SE-54), representing 98.25% of the total oil content. Forty four constituents were identified by the second column (CW20M), representing 98.12% of the total oil content. The major components detected were as follows: linalyl acetate 56.88%, linalool 20.75%, germacrene D 5.08% and β-caryophyllene 3.41%. The analyzed oil sample belongs to the group of clary sage oils of linalyl acetate/linalool chemotype (Georgiev & Stoyanova, 2006; Kuźma et al., 2009; Sharopov & Setzer, 2012).

Table 1. *Chemical composition of S. sclarea L. essential oil from Bulgaria*.

№	Compound	RI[a]	%	RI[b]	%
1	Isoamyl alcohol	727	0.01	1178	0.01
2	*trans*-2-Hexenal	849	0.04	1203	0.03
3	*cis*-3-Hexenol	852	0.29	1351	0.31
4	*trans*-2-Hexenol	861	0.14	1374	0.20
5	Hexanol	863	0.11	1326	0.08
6	α-Pinene	941	0.01	1015	0.02
7	1-Octen-3-ol	977	0.06	1421	0.07
8	Sabinene	-	-	1109	0.01
9	β-Pinene	986	0.02	1100	0.03
10	3-Octanone	-	-	1236	0.02
11	Myrcene	992	0.48	1142	0.48
12	trans-Dehydroxylinalool oxide	995	0.04	1194	0.04
13	*cis*-3-Hexenyl acetate	1003	0.01	1289	0.02
14	*cis*-Dehydroxylinalool oxide	1010	0.05	1222	0.04
15	α-Terpinene	-	-	1162	0.01
16	*p*-Cymene	1030	0.02	1254	0.03
17	Limonene	1036	0.2	1183	0.20
18	*cis*-β-Ocimene	1038	0.21	1213	0.19
19	*trans*-β-Ocimene	1050	0.4	1228	0.39
20	*trans* -Linalool oxide	1078	0.01	1417	0.03
21	*cis*-Linalool oxide	1094	0.03	1444	0.03
22	Terpinolene	1096	0.13	1259	0.11
23	Linalool	1104	20.75	1522	20.49
24	Nerol oxide	1160	0.03	-	-
25	Terpinen-4-ol	1189	0.04	-	-
26	*cis*-3-Hexenyl butanoate	1192	0.03	-	-
27	α-Terpineol	1201	2.64	1672	2.58
28	γ-Terpineol	1205	0.05	-	-
29	Linalyl formate	1222	0.22	1549	0.34
30	Nerol	1234	0.44	1768	0.45
31	Linalyl acetate	1262	56.88	1534	56.04
32	Geraniol	-	-	1818	1.05
33	Neryl formate	1284	0.05	1634	0.06
34	Geranyl formate	1304	0.12	1668	0.09
35	Acetoxy linalool	1339	0.04	-	-

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№	Compound	RI[a]	%	RI[b]	%
36	Terpinyl acetate	1358	0.03	-	-
37	Neryl acetate	1364	0.71	1694	0.69
38	α-Cubebene	-	-	1440	0.05
39	Geranyl acetate	1382	1.22	1726	1.51
40	α-Copaene	1396	0.87	1476	0.49
41	β-Burbonene	1407	0.1	1502	0.11
42	α-Ylangene	-	0.52	1473	0.38
43	*trans*-β-Caryophyllene	1444	3.41	1572	3.68
44	β-Copaene	1451	0.06	-	-
45	*trans*-β-Farnesene	1461	0.01	-	-
46	Aromadendrene	1464	0.03	-	-
47	α-Humulene	1478	0.17	1640	0.15
48	Germacren D	1505	5.08	1684	5.37
49	Valencene	1510	0.07	-	-
50	*trans*-α-Farnesene	1511	0.14	-	-
51	Bicyclogermacrene	1520	1.4	-	-
52	δ-Cadinene	1540	0.18	-	-
53	*cis*-Dihydroagarofurane	1549	0.02	-	-
54	Spathulenol	1602	0.2	2095	0.22
55	Caryophyllene oxide	1611	0.2	1956	0.21
56	Sclareol oxide	1922	0.07	2210	0.06
57	Sclareol	-	0.21	3085	0.18
	Total:	**98.25**		**98.12**	

RI[a]: 50 m x 0,25 mm x 1,0 μm SE-54, CS Chromatographie Service, Germany
RI[b]: 60 m x 0,25 mm x 0,25 μm CW20M, J & W Scientific, USA

As seen in Figure 1, oxygenated monoterpenes were the major group representing 84.57% of the total oil content, followed by sesquiterpenes 12.34%, monoterpenes 1.47%, oxygenated sesquiterpenes 0.61% and other compounds 1.02%.

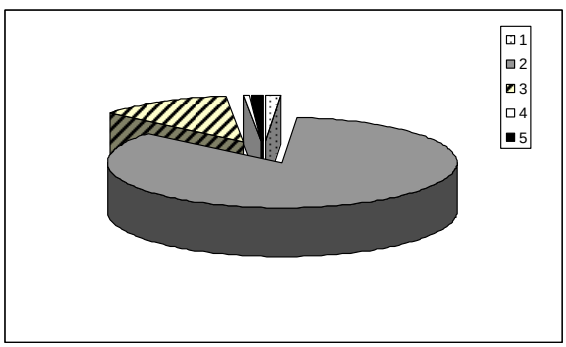

Figure 1. *Main groups of organic structures in S. sclarea essential oil: 1 – Monoterpenes, 2 – Oxygenated monoterpenes, 3 – Sesquiterpenes, 4 – Oxigenated monoterpenes, 5 – Other compounds.*

According to its chemical composition essential oil of *S. sclarea* growing in Bulgaria was comparable to commercial clary sage essential oils (Georgiev & Stoyanova, 2006).

Antifungal activity of the studied essential oil and azole antifungals fluconazole, itraconazole and ketoconazloe against clinical isolates of *Candida* species was determined by serial two fold microdillution method. The results for MICs and MFCs are shown in Table 2.

As seen (Table 2) *S. sclarea* L. essential oil demonstrated antifungal activity against all of the tested strains, belonging to the different species of genus *Candida*. The strains of species *C. albicans* and *C. krusei* were more susceptible to clary sage oil, followed by strains *C. parapsilosis*. More resistible to clary sage oil were the strains belonging to species *C. tropicalis* and *C. glabrata*. Clinical isolates were more susceptible to itraconazloe and ketoconazole than to fluconazole. MICs of clary sage oil were much higher in comparison with MICs of classical antifungals.

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Table 2. *Antifungal activity of S. sclarea L. essential oil and azole preprations against clinical isolates of Candida species*.

Substance	Species	MIC±SD (µg/ml)	MICs range	MFC±SD (µg/ml)	MFCs range
Clary sage oil	*C. albicans*	210.3±63.6	128-256	402.3±131.5	256-521
	C. tropicalis	768±295.6	512-1024	768±295.6	512-1024
	C. krusei	224±64	128-256	448±128	256-521
	C. glabrata	896±256	512-1024	1024	-
	C. parapsilosis	256	-	384±147.8	256-521
FLC	*C. albicans*	17.3±22.7	0.5-64	46.8±16.6	32-64
	C. tropicalis	6±2.8	4-8	48±22.6	32-64
	C. krusei	5±4.2	2-8	48±22.6	32-64
	C. glabrata	40±33.9	16-64	64	-
	C. parapsilosis	6±2.8	4-8	48±22.6	32-64
IT	*C. albicans*	0.4±0.6	0.125-2	29.5±6	16-32
	C. tropicalis	0.4±0.2	0.25-0.5	16	-
	C. krusei	0.4±0.2	0.25-0.5	16	-
	C. glabrata	1.3±0.1	0.5-2	32	-
	C. parapsilosis	0.2±0.1	0.125-0.5	24±11.3	16-32
KT	*C. albicans*	4±4.4	0.5-16	22.2±9.9	8-32
	C. tropicalis	3±1.4	2-4	32	-
	C. krusei	2.5±2.1	1-4	24±11.3	16-32
	C. glabrata	5±4.2	2-8	32	-
	C. parapsilosis	3±1.41	2-4	24±11.3	16-31

Table 3. *Antifungal activity of linalool and linalyl acetate against clinical isolates of Candida species*.

Substance	Species	MIC±SD (µg/ml)	MICs range	MFC±SD (µg/ml)	MFCs range
Linalool	*C. albicans*	804.6±262.9	512-1024	914.3±218	512-1024
	C. tropicalis	1536±591.2	1024-2048	1792±512	1024-2048
	C. krusei	896±256	512-1024	896±256	512-1024
	C. glabrata	1792±512	512-1024	1024	-
	C. parapsilosis	1024	-	1024	-
Linalyl acetate	*C. albicans*	384±132.8	256-512	731.4±262.9	512-1024
	C. tropicalis	1024	-	1280±512	10248-2048
	C. krusei	512	-	640±256	512-1024
	C. glabrata	895±256	512-1024	1536±591.2	1024-2048
	C. parapsilosis	512	-	1024	-

The major advantage of clary sage essential oil in comparison with tested antifungals was the fact that the essential oil was active against fluconazole, ketoconazole and itraconazole resistant strains of *C. albicans* and *C. glabrata*.

To clarify the influence of essential oil chemical composition on the anticandidial activity, the MICs and MFCs values of major pure compounds linalool and linalyl acetate against studied strains were determined. The results are shown in Table 3.

As seen (Table 3) pure compounds linalool and linalyl acetate demonstrated weaker antifungal activity against clinical isolates of *Candida* species in comparison with essential oil of *S. sclarea*. Essential oil *S. sclarea* is a mixture of more than 50 different chemical compounds and probably

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some of the major substances, in particular linalool and linalyl acetate, revealed synergic antimicrobial action against microbial cells. Of course synergic effect of some minor compounds cannot be excluded without additional experiments. Analogy with antimicrobial susceptibility determined against clary sage essential oil (Table 1), the tested strains belonging to species *C. albicans* were more susceptible to linalool and linalyl acetate, followed by *C. krusei*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata*.

On the other hand between both tested substances, linalyl acetate demonstrated stronger anticandidial activity in comparison with linalool, which means that esterification of linalool, increased its antifungal activity. Dorman and Dean (Dorman & Deans, 2000) also published that esters characterized with increased antimicrobial activity in comparison with pure compounds, which is in accordance with the results obtained in a present study.

Conclusion

The studied essential oil of *S. sclarea*, growing in Bulgaria belongs to the group of linalyl acetate/linalool chemotype. The essential oil demonstrates antifungal activity against clinical isolates of strains belonging to five different species of genus *Candida – C. albicans, C. tropicalis, C. krusei, C. glabrata* and *C. parapsilosis*. Essential oil of *S. sclarea* demonstrated stronger anticandidial activity in comparison with linalool and linalyl acetate. Antifungal activity of studied essential oil and pure compounds depends on its chemical composition and structures. The obtained results revealed possibilities for application of essential oil of S. sclarea, growing in Bulgaria, not only as flavouring agent, but even as natural product with pharmacological importance.

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