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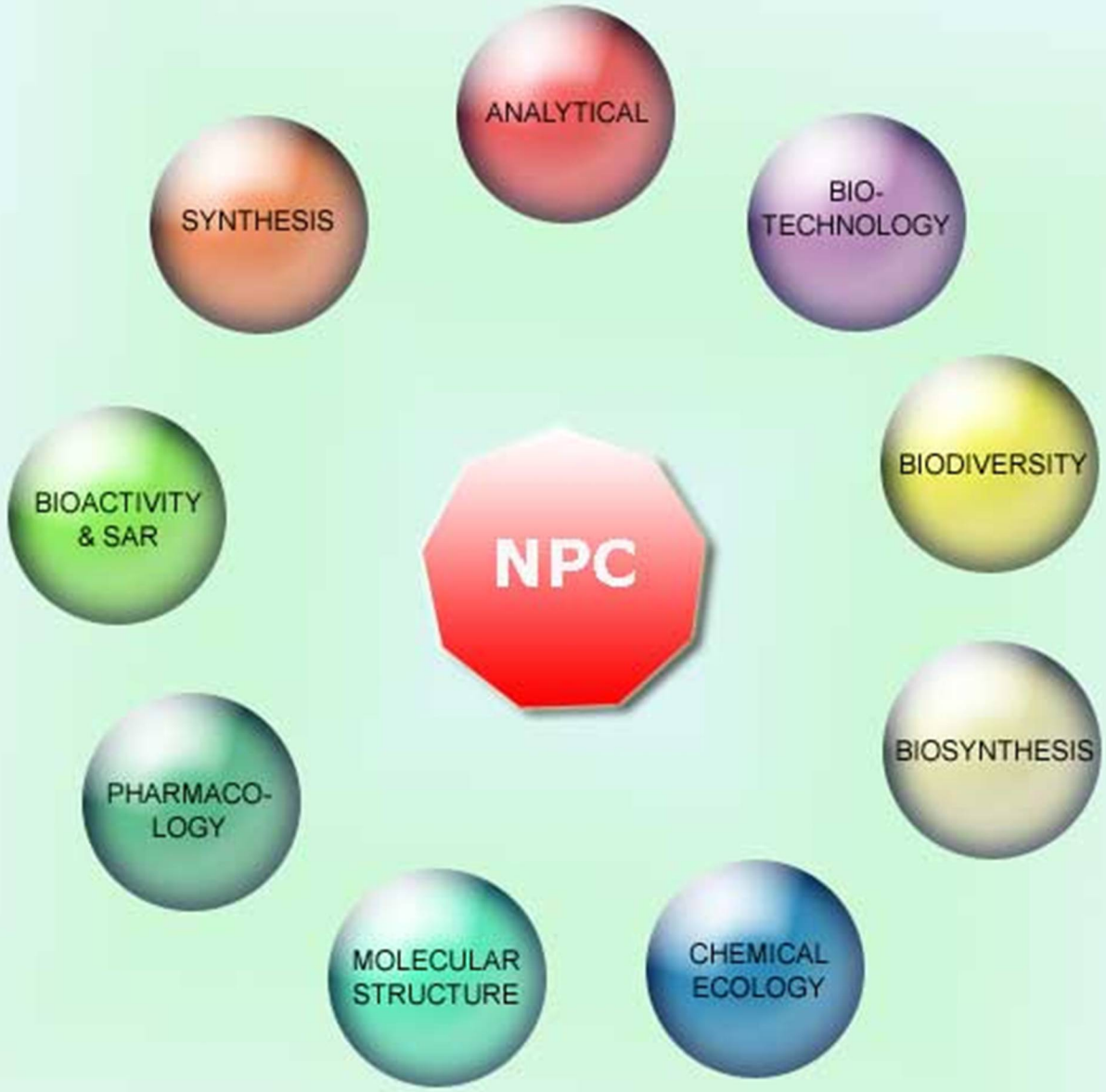

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Chemical Composition and Antimicrobial Activity of Essential Oil of Different Parts of *Seseli rigidum*

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The chemical composition and antimicrobial activity of the essential oil of the Balkan endemic species *Seseli rigidum* Waldst. & Kit. (Apiaceae) was investigated. The monoterpene α-pinene was predominant in the volatile oil from aerial parts (57.4%) and fruit (23.3%). In the essential oil of the aerial parts limonene (6.7%), camphene (5.8%) and sabinene (5.5%) were also present in high amounts, and in the fruit oil, β-phellandrene (17.4%) and sabinene (12.9%). On the contrary, the root essential oil was composed almost entirely of the polyacetylene falcarinol (88.8%). The antimicrobial activity of the root essential oil was significant against *Staphylococcus aureus*, *S. epidermidis*, *Micrococcus luteus* and *Enterococcus faecalis* (MICs 6.25-25.00 μg/mL). Volatile constituents from the root strongly inhibited the growth of methicillin-resistant strains of *S. aureus* (MICs 6.25-50.00 μg/mL). Anti-staphylococcal activity can be attributed to the main volatile constituent of *S. rigidum* root, falcarinol.

Keywords: *Seseli rigidum*, Essential oil, Falcarinol, Antimicrobial activity.

Bacteria are extremely adept at acquiring resistance and there is a constant need for new compounds with new mechanisms of action to circumvent this resistance. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the best known species of bacteria responsible for many hospital acquired [1] and community acquired infections that has disseminated rapidly over the last decade in industrialized regions [2]. This bacterium is commonly responsible for wound-related infections and life-threatening conditions, such as bacteraemia, necrotising pneumonia and endocarditis. MRSA strains are characterized by acquisition of β-lactam resistance, particularly to methicillin and oxacillin [1]. Besides resistance to all β-lactam antibiotics (penicillins and cephalosporins), it has also been detected that MRSA strains are developing resistance to other commonly used antibiotics for treatment of staphylococcal infections making them a serious therapeutic problem [3]. Since development of new antimicrobial agents depends on complex methods of classical and combinatorial synthesis of antibiotics, herbal medicines become one of the most promising sources for discovering novel antimicrobial compounds [4].

Seseli rigidum Waldst. & Kit. (Apiaceae) is an herbaceous perennial plant native to the Balkan Peninsula [5]. Some *Seseli* species are used in traditional medicine as anti-inflammatory agents, and for their carminative, stomachic and anthelmintic properties [6]. The essential oils of different *Seseli* species showed antimicrobial [7,8] and antifungal activity [9]. Ethyl acetate extracts of *Seseli* species from Turkey exhibited anti-inflammatory and antinociceptive properties [6]. *S. rigidum* flower essential oil demonstrated moderate antimicrobial and low antioxidant effects [10a,b]. There are no data about the chemical composition of *S. rigidum* root oil. Antimicrobial activity of essential oils of *S. rigidum* root, aerial parts and fruit was not previously investigated and this is the first report of the anti-staphylococcal effect of the essential oils of different parts of *S. rigidum*.

Root, aerial parts and fruit yielded 0.1%, 0.8% and 2.0% of volatile oil, respectively. Sixty-two compounds (Table 1) were identified, representing 94.8-99.7% of the total oil. Monoterpenes were predominant in the essential oil of the aerial parts (93.2%) and in the fruit oil (71.6%), while in the root oil they were presented only in small amounts (0.8%). The essential oil of the root almost completely consisted of the polyacetylene falcarinol (88.8%). On the other hand, falcarinol represented only 3.0% of the fruit oil and was absent in the oil of the aerial parts.

α-Pinene (57.4%), limonene (6.7%), camphene (5.8%), sabinene (5.5%) and myrcene (5.0%) were major compounds in the oil of the aerial parts. Previous results for the essential oil of the aerial parts of *S. rigidum* showed that α-pinene (53.3%), limonene (10.0%), germacrene D (9.3%) and myrcene (6.0%) were predominant [11].

The fruit oil was characterized by high amounts of α-pinene (23.3%), β-phellandrene (17.4%) and sabinene (12.9%). Earlier investigations showed that α-pinene and sabinene were main compounds in the fruit oil, but there was no identification of the sesquiterpenes [12]. In our sample sesquiterpenes represented 21.3% of the fruit oil: *E*-caryophyllene (3.7%), germacrene B (3.6%) and germacrene D (3.2%) were in significant amounts.

On the contrary, the root oil contained falcarinol (88.8%) as its main compound. Monoterpenes were abundant in the oils of the fruit and aerial parts: α-pinene, camphene, sabinene, limonene were present only in low quantities. Myrcene, β-phellandrene, germacrene D and germacrene B were absent from the root oil.

Antimicrobial activities of the three essential oils of *S. rigidum* were investigated against laboratory control strains of *Staphylococcus aureus*, *S. epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis*,

Table 1: Composition of the essential oils of *S. rigidum* root, aerial parts and fruit.

Constituents	RI[a]	Content (%)				RI	Content (%)		
		R[b]	A[c]	F[d]			R	A	F
Tricyclene	924	-	0.1	t[e]	β-Elemene	1395	t	0.3	0.5
α-Thujene	927	-	0.1	t	*E*-Caryophyllene	1423	t	0.7	3.7
α-Pinene	938	0.2	57.4	23.3	2,5-Dimethoxy-*p*-cymene	1426	0.3	-	t
Camphene	951	t	5.8	2.3	α-Humulene	1457	t	t	0.1
Sabinene	975	t	5.5	12.9	*E*-β-Farnesene	1459	-	-	1.0
β-Pinene	979	t	3.1	2.1	γ-Muurolene	1475	t	-	0.6
Myrcene	992	-	5.0	3.4	Germacrene D	1485	-	1.8	3.2
n-Octanal	1003	0.3	0.1	t	β-Selinene	1489	0.1	t	t
α-Phellandrene	1006	-	0.1	3.9	α-Selinene	1498	0.2	t	-
α-Terpinene	1018	-	0.1	t	Bicyclogermacrene	1500	-	0.3	1.0
p-Cymene	1025	t	0.3	1.2	α-Muurolene	1502	0.3	-	-
Limonene	1030	t	6.7	t	Germacrene A	1509	-	0.3	0.2
β-Phellandrene	1031	-	t	17.4	δ-Amorphene	1512	0.3	-	-
Z-β-Ocimene	1037	-	0.2	t	δ-Cadinene	1526	0.4	t	0.4
γ-Terpinene	1059	t	0.3	2.2	Germacrene B	1561	-	0.3	3.6
Terpinolene	1090	-	0.2	t	Spathulenol	1581	0.1	0.6	t
Linalool	1101	t	0.6	t	Caryophyllene oxide	1587	1.0	1.3	0.6
α-Campholenal	1128	t	0.4	t	Salvial-4(14)-en-1-one	1598	0.2	t	t
trans-Pinocarveol	1140	t	0.5	t	Carotol	1602	-	-	2.3
cis-Verbenol	1142	t	0.2	t	Humulene epoxide II	1613	t	0.2	t
trans-Verbenol	1146	t	0.7	t	β-Oplopenone	1622	-	t	0.5
Pinocarvone	1164	t	0.2	t	Isospathulenol	1636	t	0.2	-
p-Mentha-1,5-dien-8-ol	1168	-	0.3	t	Muurola-4,10(14)dien-1-β-ol	1637	0.1	t	3.1
Terpinen-4-ol	1179	t	0.9	0.8	3-Butyl phthalide	1655	0.2	-	-
α-Terpineol	1192	t	0.2	t	3-*Z*-Butylidene phthalide	1675	0.2	-	-
Myrtenal	1199	t	0.4	t	α-Cadinol	1658	t	0.2	t
Verbenone	1212	t	0.2	t	Falcarinol	2036	88.8	-	3.0
trans-Carveol	1220	-	0.2	t	Linoleic acid	2134	0.8	-	-
2-*E*-Decenal	1262	0.1	t	-					
Bornyl acetate	1289	0.3	3.5	2.1	Monoterpene hydrocarbons		0.2	84.9	68.7
2*E*,4*Z*-Decadienal	1295	0.3	-	-	Oxygenated monoterpenes		0.6	8.3	2.9
2*E*,4*E*-Decadienal	1318	0.6	-	-	Sesquiterpene hydrocarbons		1.3	3.9	14.8
Daucene	1383	t	-	0.5	Oxygenated sesquiterpenes		1.4	2.5	6.5
β-Bourbonene	1388	t	0.2	t		Other	91.3	0.1	3.0
						Total	94.8	99.7	95.9

[a] Retention indices on HP-5 MS column; [b] essential oil of root; [c] essential oil of aerial parts; [d] essential oil of fruit; [e] trace (<0.1%).

Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, *Pseudomonas aeruginosa* and four methicillin-resistant strains of *S. aureus*.

Only data for the antimicrobial activity of the root essential oil are represented in Table 2, because the essential oil of the aerial parts and fruit showed low effects against the laboratory control strains and were not active against the investigated MRSA strains.

The essential oil of *S. rigidum* root showed significant antimicrobial effect against Gram-positive bacteria: *S. aureus*, *S. epidermidis*, *M. luteus* and *E. faecalis* (Table 2). Minimal inhibitory concentrations (MICs) were in the range 6.25-25.00 μg/mL. Among the tested bacteria the most sensitive strain to *S. rigidum* root oil was *S. epidermidis*, with a MIC value 6.25 μg/mL. Antimicrobial effect is considered significant if MIC values of the investigated extract or essential oil are below 100 μg/mL [13]. Essential oils as typical lipophiles pass through the bacterial membrane, disrupt its structure and permeabilize it, which consecutively leads to reduction of membrane potential, collapse of the proton pump, depletion of the ATP pool, leakage of macromolecules and lysis. Essential oil can also coagulate the cytoplasm and damage the lipids and proteins in bacteria [14].

Essential oils of aerial parts and fruit exerted low inhibitory effects against the Gram-positive bacteria *S. aureus*, *S. epidermidis*, *M. luteus* and *E. faecalis* (MICs 200 μg/mL). Such a low efficiency could be explained by the low inhibitory effect of α-pinene (MIC >900 μg/mL) [15], one of the main volatile compounds in the aerial parts and fruit.

The investigated oils did not inhibit the growth of the Gram-positive *Bacillus subtilis* and Gram-negative *E. coli*, *K. pneumoniae* and *P. aeruginosa* (MICs >200 μg/mL). Gram-negative bacteria have a complex cell wall, an outer membrane that is an effective barrier to different compounds and a set of multidrug resistance pumps that extrude toxins across the outer membrane [16]. Differences in the cell wall structure between Gram-positive and Gram-negative bacteria are responsible for making these bacteria more resistant to commonly used antibiotics as well as to essential oil constituents compared to Gram-positive bacteria [4].

The essential oil of the root effectively inhibited the growth of methicillin-resistant strains of *S. aureus* (Table 2). MIC values were in the range 6.25-50.00 μg/mL. Root oil was more active against MRSA strain N^o 4 than against the non-resistant ATCC strain of *S. aureus*. Previous studies also demonstrated that certain plant natural products are more active against multidrug resistant strains than sensitive strains [16,17].

The antimicrobial and anti-staphylococcal activity of *S. rigidum* root oil can be attributed to its high falcarinol content. Previous investigations showed that falcarinol from bark of *Oplopanax horridus* inhibited the growth of *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *Mycobacterium tuberculosis, M. avium* and the yeast *Candida albicans* [18], and falcarinol from the root of *Levisticum officinale* exhibited activity against *M. fortuitum* and *M. aurum*. The polyacetylene dehydrofalcarindiol, with a double bond instead of a terminal methyl group compared with falcarinol, isolated from *Artemisia monosperma*, showed no antimycobacterial effect, which indicates that the terminal methyl group in falcarinol is essential for activity [19]. Falcarinol is also highly cytotoxic against numerous

Table 2: Antimicrobial activity of *S. rigidum* root essential oil against different laboratory control strains of bacteria and methicillin-resistant *S. aureus* strains, as minimal inhibitory concentrations (MICs).

Microorganisms	MIC (µg/mL)						
	R[a]	Amp[b]	Ami[c]	Oxa[d]	Tobra[e]	FA[f]	Vanco[g]
Staphylococcus aureus ATCC 25923	12.50	0.5	n.t.	n.t.	n.t.	n.t.	n.t.
Staphylococcus epidermidis ATCC 12228	6.25	1	n.t.	n.t.	n.t.	n.t.	n.t.
Micrococcus luteus ATCC 9341	25.00	0.5	n.t.	n.t.	n.t.	n.t.	n.t.
Enterococcus faecalis ATCC 29212	25.00	1	n.t.	n.t.	n.t.	n.t.	n.t.
Bacillus subtilis ATCC 6633	>200	2	n.t.	n.t.	n.t.	n.t.	n.t.
Escherichia coli ATCC 25922	>200	4	2	n.t.	n.t.	n.t.	n.t.
Klebsiella pneumoniae NCIMB 9111	>200	4	2	n.t.	n.t.	n.t.	n.t.
Pseudomonas aeruginosa ATCC 27853	>200	8	4	n.t.	n.t.	n.t.	n.t.
MRSA N° 4	6.25	>16 ^{R}	<16 ^{S}	<4 ^{S}	<16 ^{S}	<32 ^{S}	<32 ^{S}
MRSA N° 16	25.00	>16 ^{R}	<16 ^{S}	>4 ^{R}	>16 ^{R}	<32 ^{S}	<32 ^{S}
MRSA N° 50	50.00	>16 ^{R}	>16 ^{R}	>4 ^{R}	>16 ^{R}	<32 ^{S}	<32 ^{S}
MRSA ATCC 43300	25.00	>16 ^{R}	>16 ^{R}	>4 ^{R}	>16 ^{R}	<32 ^{S}	<32 ^{S}

[a]essential oil of root; [b] ampicillin; [c] amikacin; [d] oxacillin; [e] tobramycin; [f] fusidic acid; [g] vancomycin; [S] sensitive (determined by automated system for susceptibility testing); [R] resistant (determined by automated system for susceptibility testing).

cancer cell lines [20] and shows anti-inflammatory properties [21]. Its bioactivity is probably due to its ability to form an extremely stable carbocation and to act as a very reactive alkylating agent towards mercapto and amino groups in proteins and other biomolecules [20].

Activity against methicillin resistant *S. aureus* indicates that falcarinol should be further investigated as an antimicrobial agent.

Experimental

Plant material: Root and aerial parts, before flowering, of *Seseli rigidum* were collected in June 2010, and fruits in September 2010, in Brdjanska gorge in west Serbia. Herbarium specimens were deposited at the Herbarium of the Faculty of Pharmacy in Belgrade (3228HFF, 3240HFF). Plant material was air dried and ground to coarse powder. Essential oils were obtained by hydrodistillation in a Clevenger-type apparatus according to the procedure given in the European Pharmacopoeia 7.0 [22].

Essential oil analysis: Volatile constituents were determined by GC and GC-MS. GC analysis was performed on an Agilent 6890N GC system equipped with 5975 MSD and FID, using a HP-5 MS column (30 m x 0.25 mm x 0.25 µm). Injection volume was 2 µL and the injector temperature was 200°C with a 10:1 split ratio. Helium was the carrier gas and its flow rate was 1.0 mL/min (constant flow mode). Column temperature was linearly programmed in the range 60-280°C at a rate of 3°C/min and held at 280°C for 5 min. The transfer line was heated at 250°C. The FID detector temperature was 300°C. EI mass spectra (70 eV) were acquired in the m/z range 35-550. The retention indices were experimentally determined using *n*-alkanes (C_8-C_{20} and C_{21}-C_{40}) injected after the essential oil, under the same chromatographic conditions. The identification of the compounds was based on the comparison of their retention indices (RI), their retention times (t_R) and mass spectra with those obtained from authentic samples and/or the NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) software, Wiley libraries, Adams data base and literature [23]. Relative percentages of the identified compounds were computed from the GC-FID peak area.

Bacterial strains and culture media: Antimicrobial activity of *S. rigidum* essential oils was tested against 9 laboratory control strains of bacteria: *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 9341, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* NCIMB 9111, *Pseudomonas aeruginosa* ATCC 27853, methicillin-resistant *S. aureus* (MRSA) ATCC 43300 and three clinical isolates of MRSA obtained from sputum (MRSA strain N° 4), abdominal wound (MRSA strain N° 16) and endotracheal tube (MRSA strain N° 50). Identification of the isolates and methicillin resistance were determined by VITEK 2 test cards GP and AST-P580 (bioMérieux, France) and confirmed by PCR for *nuc* [24] and *mec*A [25] genes. One laboratory control strain of methicillin-resistant *S. aureus* ATCC 43300 was used as a positive control.

Clinical isolates of MRSA were stored at -70°C in brain heart infusion broth (BHI; Lab M Limited, UK) with the addition of 10% sterile glycerol. Prior to experiment, bacteria were defrosted, inoculated on tryptic soy agar (TSA; Lab M Limited) and cultivated in aerobic conditions for 18-24h at 35°C.

Broth microdilution method: Minimal inhibitory concentrations (MICs) of *S. rigidum* essential oils were determined by a broth microdilution method according to the Clinical and Laboratory Standards Institute [26]. Shortly, one colony of the overnight cultures of bacterial strains was diluted in saline in order to adjust the turbidity of the bacterial suspension to 0.5 McFarland standard (approximately 10^8 CFU/mL). Serial dilutions of essential oils (in the range of 6.25-400 µg/mL) were prepared in fresh Müller-Hinton broth with addition of triphenyltetrazolium chloride (0.05%) as a growth indicator, and 180 µL of each dilution was poured in triplicate into a 96-well microtiter plate. Twenty µL of a previously prepared bacterial suspension was added to each well. Positive growth controls of each strain (bacteria in medium without presence of essential oil) were incubated under the same conditions. The negative control for each plate was medium solely. After incubation for 24 h at 35°C in aerobic conditions, MIC was identified as the lowest concentration of the oil showing no visible growth of tested microorganisms. Each test was repeated 3 times.

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Review/Account

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