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Chemical Composition and Biological Activity of Essential Oils from Wild Growing Aromatic Plant Species of *Skimmia laureola* and *Juniperus macropoda* from Western Himalaya

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The Himalayan region is very rich in a great variety of medicinal plants. In this investigation the essential oils of two selected species are described for their antimicrobial and larvicidal as well as biting deterrent activities. Additionally, the odors are characterized. Analyzed by simultaneous GC-MS and GC-FID, the essential oils' chemical compositions are given. The main components of *Skimmia laureola* oil were linalool and linalyl acetate whereas sabinene was found as the main compound for *Juniperus macropoda* essential oil. Antibacterial testing by agar dilution assay revealed highest activity of *S. laureola* oil against all tested bacteria, followed by *J. macropoda* oil. Antifungal activity was evaluated against the strawberry anthracnose causing plant pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides*. *Juniperus macropoda* essential oil indicated higher antifungal activity against all three pathogens than *S. laureola* oil. Both essential oils showed biting deterrent activity above solvent control but low larvicidal activity.

Keywords: *Skimmia laureola*, *Juniperus macropoda*, Essential oil, Antifungal activity, Antibacterial activity, Biting deterrent activity, Larvicidal activity.

In the Himalayan region most of the local population, living apart from the cities, depend on traditional medicine mainly using wild species [1]. *Skimmia laureola* (DC.) Siebold & Zucc. ex Walp. (*Rutaceae*), an evergreen shrub or tree, is native in India and China. The leaves are burned and inhaled against fever and body pain in traditional medicine [2]. *Juniperus macropoda* Boiss. (syn. of *Juniperus polycarpos* K. Koch var. *seravschanica* (Kom.) Kitam., *Cupressaceae)*, commonly known as the Indian juniper or Himalayan pencil cedar, is an evergreen tree native on the Indian subcontinent (India, Pakistan) as well as in western and middle Asia. In Himalaya it can be found at altitudes up to 4500 m above sea level [3]. The ethnic communities inhabiting high mountain areas use this plant as fuels and timber wood, for religious purposes and in health care. Among the Buddhists, the plant is regarded as a sacred tree [4, 5].

In the present investigation the essential oils (EOs) of these two diverse but typical Himalayan plants collected wild in the western Himalayan region (India) were analyzed using simultaneous GC-FID and GC-MS. Tables 1 and 2 show the results of the quantitative and qualitative analyses.

A total of 41 compounds, accounting for 97.5%, were identified from *S. laureola* EO. It possessed a high amount of linalool (34.9%) and linalyl acetate (26.7%) followed by α-terpineol (12.8%) and geranyl acetate (6.6%) (Table 1). This mainly corresponds with the findings from other investigations on Indian samples [6, 7]. In another study a large variation in the oils' contents of linalyl acetate and linalool depending on the harvesting season of the plants was reported [8].

The EO of green needles and thin stem parts from *J. macropoda* contained mainly sabinene (27.5%) of a total of 67 compounds (85.4% of the oil). Cedrol (14.1%), terpinen-4-ol (9.4%) and p-cymene (4.2%) were also detected in appreciable amounts (Table 2). A comparative study on *J. macropoda* leaf oil collected from plants growing in Garhwal regions (Uttranchal, India) demonstrated high variation in oil composition: Whereas in one oil sample β-elemene (42.5%) was the main constituent, in the other oil sample α-thujone (22.6%) was prominent. Generally only 19 constituents were similar in both oils. Sabinene was found only in one sample (5.8%) [3]. In another investigation on geographic variation in leaf EO of *J. polycarpos* in central Asia, Adams found a large variability in the constituents of the oils from Armenia, Turkmenistan, Pakistan and Kazakhstan. The amount of α-pinene ranged from 15.5% to 68.8%, whereas cedrol was found in 26.4% to traces. In all the examined oils the content of sabinene was below 1% [9]. These findings again show the dramatic influences of climatic and local factors on EO compositions [10, 11].

The odor of *S. laureola* EO was described as freshly bergamot-like with a soft herbaceous lavender and somewhat woody note. Olfactory evaluation of *J. macropoda* oil revealed a terpene-like, coniferous, harsh and fatty odor.

Table 1: Composition of *S. laureola* oil.

No. of Compds	Substance	RT[min.]	RI[#]	%Area$_{FID}$
1	α-Thujene	10.87	925	0.0
2	α-Pinene	11.33	937	0.1
3	Sabinene	12.81	977	0.1
4	6-Methyl-5-hepten-2-one	12.9	980	0.0
5	β-Pinene	13	982	0.0
6	Myrcene	13.37	992	0.1
7	Dehydro-1,8-cineole	13.48	994	0.0
8	*cis*-3-Hexenyl acetate	13.61	998	0.0
9	*p*-Cymene	14.87	1027	0.1
10	Limonene	15.07	1032	0.3
11	1,8-Cineole	15.21	1035	1.3
12	(*E*)-β-Ocimene	15.71	1047	0.0
13	γ-Terpinene	16.41	1062	0.0
14	Octanol	16.59	1066	0.0
15	*cis*-Linalool oxide (furanoid)	16.97	1076	0.7
16	*trans*-Linalool oxide (furanoid)	17.66	1092	0.6
17	Linalool	18.18	1104	34.9
18	Nerol oxide	20.5	1155	0.0
19	*cis*-Linalool oxide (pyranoid)	21.32	1174	0.1
20	*trans*-Linalool oxide (pyranoid)	21.5	1178	0.1
21	Terpinen-4-ol	21.73	1183	0.2
22	*p*-Cymen-8-ol	22.07	1190	0.1
23	*cis*-3-Hexenyl butyrate	22.22	1194	0.4
24	α-Terpineol	22.37	1197	12.8
25	Octyl acetate	22.65	1204	0.1
26	Nerol	23.9	1231	1.5
27	Neral	24.45	1244	0.3
28	Linalyl acetate	25.1	1258	26.7
29	Geraniol	25.2	1259	4.1
30	Geranial	25.73	1273	0.5
31	Neryl formate	26.16	1282	0.2
32	Geranyl formate	27.07	1303	0.6
33	5-Acetoxylinalool	28.7	1341	0.4
34	α-Terpinyl acetate	29.23	1353	1.4
35	Neryl acetate	29.72	1365	2.8
36	8-Hydroxylinalool	29.94	1370	0.1
37	Geranyl acetate	30.54	1384	6.6
38	Carvon hydrate	32.54	1432	0.2
39	*trans*-Nerolidol	37.91	1567	0.1
40	Spathulenol	38.76	1589	0.1
41	Elemol	42.38	1686	0.2
	sum			97.5

[#] RI on 50 m x 0.25 mm x 0.25 mm SE-52 column. tr. = trace (<0.05%).

Table 2: Composition of *J. macropoda* oil.

No. of Compds	Substance	RT[min.]	RI[#]	%Area$_{FID}$
1	Tricyclene	10.82	924	tr.
2	α-Thujene	11.05	930	1.4
3	α-Pinene	11.35	938	2.7
4	α-Fenchene	11.86	952	tr.
5	Camphene	11.94	954	0.1
6	Sabinene	12.85	978	27.5
7	β-Pinene	13.02	982	0.4
8	Myrcene	13.39	992	0.1
9	Methyl 4-methylhexanoate	13.61	998	0.1
10	δ-3-Carene	14.32	1015	0.1
11	α-Terpinene	14.57	1020	0.1
12	*p*-Cymene	14.88	1028	4.2
13	Limonene	15.07	1032	1.2
14	1,8-Cineole	15.22	1035	0.2
15	γ-Terpinene	16.34	1061	0.3
16	*cis*-Sabinene hydrate	16.77	1071	2.6
17	*cis*-Linalool oxide	16.9	1074	0.1
18	*trans*-Linalool oxide	16.98	1076	0.1
19	Terpinolen	17.66	1092	0.1
20	Linalool	18.09	1102	0.3
21	*trans*-Sabinene hydrate	18.17	1103	2.7
22	α-Pinene oxide	18.5	1112	0.1
23	*cis*-Rose oxide	18.58	1113	0.1
24	β-Thujone	18.97	1121	0.1
25	*cis*-*p*-Menth-2-en-1-ol	19.2	1126	0.8
26	α-Campholenal	19.37	1130	0.1
27	4-Hydroxy-4-methylcyclohex-2-enone	19.46	1132	0.3
28	*trans*-*p*-Mentha-2,8-dien-1-ol	19.78	1139	0.1
29	*trans*-*p*-Menth-2-en-1-ol	19.98	1144	0.4
30	*trans*-Sabinol	20.02	1145	0.5
31	*cis*-Verbenol	20.28	1150	0.2
32	Sabina ketone	20.83	1163	1.0
33	*trans*-Pinocamphone	20.99	1166	0.1
34	Sabinol (isomer)	21.31	1173	0.4
35	*cis*-Pinocamphone	21.62	1180	0.1
36	Terpinen-4-ol	21.75	1183	9.4
37	*p*-Cymen-8-ol	22.03	1189	1.0
38	Cryptone	22.15	1192	0.1
39	α-Terpineol	22.31	1196	0.3
40	*cis*-Piperitol	22.95	1210	0.1
41	Verbenone	23.06	1212	0.2
42	*cis*-Carveol	23.54	1223	0.2
43	Cuminaldehyde	23.64	1226	0.3
44	Citronellol	23.81	1229	0.7
45	Carvone	24.65	1248	0.1
46	Methyl citronellate	25.19	1260	2.8
47	Menth-2-en-1,4-diol	25.87	1276	1.2
48	Bornyl acetate	26.52	1290	0.3
49	Menth-2-en-1,4-diol (isomer)	26.71	1295	0.4
50	Cumin alcohol	26.75	1296	0.4
51	Carvacrol	27.24	1307	1.0
52	Methyl geranate	27.98	1324	0.5
53	β-Bourbonene	30.95	1394	tr.
54	α-Cedrene	32.18	1424	1.1
55	β-Cedrene	32.52	1432	0.2
56	Thujopsene	32.92	1442	0.2
57	α-Amorphene	35.58	1507	0.2
58	β-Bisabolene	35.8	1513	0.1
59	Cuparene	35.89	1515	0.2
60	γ-Cadinene	36.17	1522	0.4
61	*cis*-Calamenene	36.49	1531	0.1
62	Elemol	37.52	1557	0.2
63	*allo*-Cedrol	39.31	1603	0.8
64	Cedrol	39.79	1616	14.1
65	τ-Muurol	41.06	1651	0.1
66	τ-Cadinol	41.25	1656	0.1
67	α-Cadinol	41.58	1665	0.2
	sum			85.4

[#] RI on 50m x 0.25mm x 0.25 mm SE-52 column
tr. = trace (<0.05%)

The intense use of antibiotics noticeably resulted in the development of drug-resistant strains. In the context of the search for new natural antibiotics the interest in EOs has grown. Therefor the EOs of *S. laureola* and *J. macropoda* were tested for their antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella abony*, *Pseudomonas aeruginosa*, as well as *Candida albicans* in an agar dilution assay. In Table 3 minimum inhibitory concentrations (MICs) in μg/mL are given.

Juniperus macropoda oil showed moderate antibacterial activity against *S. aureus*, *E. coli* and *S. abony* (MICs of 1000 μg/mL, each) but it proved to be very active against *C. albicans* (MIC 250 μg/mL), which might rather be due to terpinen-4-ol than to sabinene [12, 13]. *Skimmia laureola* was highly active against *E. coli*, *S. abony* (MIC 500 μg/mL, respectively) and *C. albicans* (MIC 250 μg/mL). High antibacterial activity of Pakistani *S. laureola* EO against *S. aureus* and *E. coli* was also reported by Irshad and coworkers [14]. In another Indian study the EO of *S. laureola* collected from the region of Tehri Garhwal was very active against *E. coli* and *P. aeruginosa*, less against *S. aureus* [6]. However, in the present investigation the oil showed only moderate results against *S. aureus* (MIC 1000 μg/mL) and *P. aeruginosa* (MIC 4000 μg/mL). This discrepancy might be due to differences in test-methods, bacterial strains and plant species.

To sum up, *C. albicans* proved to be the only strains susceptible to both EOs tested in this study, whereas *P. aeruginosa* was the most resistant one.

It has been claimed that EOs exhibit stronger biological activity than their main components. In the last decade several studies have determined this contrast in antimicrobial efficiency of EOs and their main compounds [12, 13, 15, 16, 17]. All these investigations concluded that in most cases the EOs or a combination of two or more components were more effective than a single compound. Even though the character compounds of an EO can reach an amount of 85% of the oil, EOs are complex mixtures of up to 200 components (rose oil is known to consist of more than 270 compounds). Therefore secondary compounds or components that have not been identified in two-dimensional GC-MS seem to strongly influence the biological impact of EOs.

Table 3: Antimicrobial activity of *S. laureola* and *J. macropoda* EOs and reference substances (agar dilution assay, MIC in µg/mL).

Samples	*S. aureus* ATCC 6538	*E. coli* ATCC 25922	*S. abony* ATCC 6017	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
S. laureola	1000	500	500	4000	250
J. macropoda	1000	1000	1000	4000	250
Ciprofloxacin	0.25	0.15	0.25	1	-
Cefazolin	0.50	2	2	4	-
Amphotericin B	-	-	-	-	0.25
Fluconazole	-	-	-	-	0.25

Further antifungal activity of the EOs of *S. laureola* and *J. macropoda* was tested using direct bioautography assays against three *Colletotrichum* species. Bioautography techniques are used as primary screening tools to detect antifungals from natural sources [18]. Antifungal activity was evident by the presence of clear zones with a dark background where fungal mycelia or reproductive stroma were not present on the TLC plates [18]. *Skimmia laureola* and *J. macropoda* EOs were evaluated at 80 and 160 µg/spot against three fungal pathogens *C. acutatum*, *C. fragariae* and *C. gloeosporioides* (Table 4).

Table 4: Antifungal activity of *S. laureola*, *J. macropoda* and *B. persicum* EOs against three *Colletotrichum* species.

Samples	Mean fungal growth inhibition[§] (mm) ± SD					
	C. acutatum		*C. fragariae*		*C. gloeosporioides*	
	80 µg/spot	160 µg/spot	80 µg/spot	160 µg/spot	80 µg/spot	160 µg/spot
S. laureola	4.5±0.71	6.5±0.71	5.0±0.00	7.0±0.00	4.5±0.71	6.5±0.71
J. macropoda	4.5±0.71	6.0±0.00	5.0±0.00	7.5±0.71	5.0±0.00	6.5±0.71
Standard fungicides*						
Benomyl	Diffuse		21.5±0.71		Diffuse	
Captan	12±0.00		18.5±0.71		19.5±0.71	
Cyprodinil	Diffuse		Diffuse		Diffuse	
Azoxystrobin	Diffuse		24.5±1.41		Diffuse	

[§] Mean inhibitory zones ± standard deviations; * Technical grade of internal standards; were applied at 1.16 µg (benomyl), 1.2 µg (captan), 0.9 µg (Cyprodinil) and 1.61 µg (Azoxystrobin)

Both essential oils showed almost similar level of antifungal activity. In our previous antifungal studies, we evaluated two *Juniperus* EOs, *J. saltuaria* and *J. squamata*. Sabinene was the major compound for these oils. Both oils showed weak antifungal activity against *Colletotrichum* species [19]. Since the majority of the Juniper oils were rich in sabinene and the tested oils showed weak activity, it can be concluded that sabinene is not much contributing to the antifungal activity against these plant pathogens. We have not evaluated any *Skimmia* species in the antifungal assays, but we previously tested (+)-linalool and (-)-linalool [20]. Both enantiomers did not show antifungal activity at 20mg/mL concentration using direct-bioautography assays against the same three *Colletotrichum* species. However, linalool enantiomers demonstrated 50% growth inhibition of *Botrytis cinerea*, another plant pathogen fungus preferably affecting grapes, at 48 hrs using 96-well micro-dilution assays [20]. Therefore, it can be deduced that linalool is not the active compound against *Colletotrichum* species in the *S. laureola* EO. Linalyl acetate or other secondary components and their combinations seem to be conductive to the antifungal activity, which should be assessed in further bioassays in the future.

In larvicidal screening bioassays, the EO of *J. macropoda* gave 60% mortality of *Aedes aegypti* larvae whereas *S. laureola* oil was not active at the highest dose of 125 ppm. A study on the EO of *J. macropoda* fruits from India reported high larvicidal but no repellent activity against *Anopheles stephensi*, *Ae. aegypti* and *Culex quinquefaciatus* larvae [21]. In another Pakistani study the leave oil of *S. laureola* was tested in three concentrations (1, 5 and 10%) against black ants (*Lasius niger*) where the oil showed least repellent activity of all examined oils (LC_{50}=10.15µL) [22].

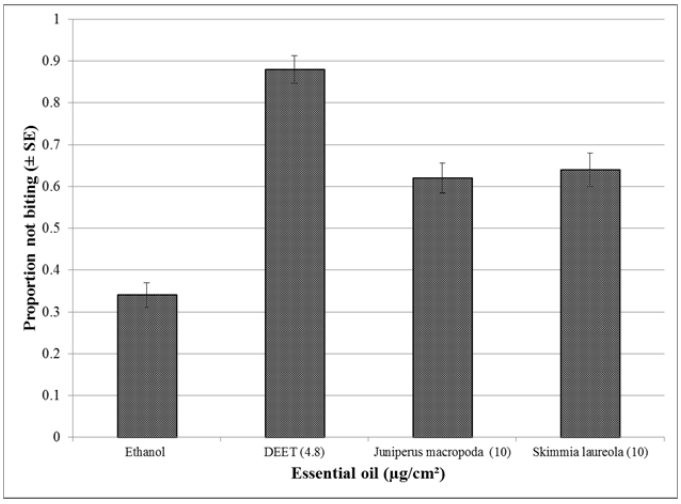

Figure 1: Proportion not biting values of *J. macropoda* and *S. laureola* essential oils against female *Ae. aegypti*. DEET, at 4.8 µg/cm^2, was used as positive control. Ethanol was used as solvent control.

In our biting deterrent bioassays, all EOs tested showed activity against *Ae. aegypti* above solvent control (ethanol) but it was significantly lower than DEET, a standard biting deterrent, which was used as a positive control (Figure 1). However, this is the first report on biting deterrent and larvicidal activity of *S. laureola* and *J. macropoda* EOs against mosquitoes.

In conclusion, the investigated EO from *S. laureola* showed rather high antibiotic but moderate antifungal activity. *J. macropoda* EO was somewhat less active than *S. laureola* in all tested conditions. So the use of both plants in traditional medicine by the isolated living population in the Himalayan region is more than justified and the oils' biological impact confirmed. Furthermore, according to their above described properties the EOs examined in this study showed promising activity as alternative natural fungicides and antibiotics.

Experimental

Plant material and isolation procedure: Leaves and aerial parts of *S. laureola* were collected from wild from Dalhousie area in Chamba (western Himalaya) at 2000 m in July/August 2010. Green needles along with thin stem parts of *J. macropoda* were collected from alpine Himalayan region (3000 m) at the same time.

Plant samples were identified by the taxonomist of the Biodiversity division of the IHBT, Palampur. The voucher specimens (*S. laureola* 1055, *J. macropoda* 7303,) were deposited in the herbarium-PLP of the IHBT, Palampur. Leaves and aerial parts (4 kg) of *S. laureola* as well as needles and stem parts (5.2 kg) of *J. macropoda* were air dried in the shade at 25°C. Hydrodistillation described in [18] yielded in colorless oils: 0.25% for *S. laureola* and 1.2% for *J. macropoda* on fresh wt. basis, respectively.

Essential oil analysis: GC-FID and GC-MS analyses and compound identification were performed according to [18].

Olfactory evaluation: For olfactory evaluation, one droplet of each essential oil sample was applied onto commercially available paper blotters. Each sample was examined described in [18].

Antimicrobial testing: The antimicrobial effects were tested according to [18] against the Gram-positive bacteria *S. aureus* (ATCC 6538), as well as the Gram-negative bacteria *E. coli* (ATCC

25922), *S. abony* (ATCC 6017) and *P. aeruginosa* (ATCC 27853). Additionally antifungal testing against *C. albicans* ATCC 10231 was performed [18].

***Antifungal testing*:** Direct bioautography techniques were used to detect antifungal activity against *Colletotrichum fragariae*, *C. acutatum* and *C. gloeosporioides* suspensions described in [18].

Mosquitoes: *Aedes aegypti* larvae from a laboratory colony were used as described in [18].

Mosquito Biting Bioassays: Experiments were conducted by using a six-celled *in vitro* Klun and Debboun (K&D) module bioassay system for quantitative evaluation of biting deterrent properties of the EOs of *J. macropoda* and *S. laureola*. Details of this bioassay are described in [23]. Treatments were replicated 10 times.

Larvicidal Bioassays: Bioassays were conducted to test essential oils of *J. macropoda* and *S. laureola* for their larvicidal activity against *Ae. aegypti* by using the bioassay system described by Pridgeon et al. [24]. Details of this bioassay and of statistical analyses are described in [23].

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