JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue

JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)

An International Scholarly Open Access, Peer-reviewed, Refereed Journal

COMPOSITION AND ANTIFUNGAL ACTIVITY OF VOLATILES FROM LIMNOPHILA REPENS BENTH.

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ABSTRACT : Essential oil from the aquatic weed *Limnophila repens* was obtained by hydrodistillation and analysed by GC/MS and olfactometry. The major compounds obtained were of terpinen-4-ol followed by sabinene, terpinolene, oct-1-en-3-ol, trans-β-farnesene, and γ-terpinene. The antifungal activity of the oil against three plant pathogens *Pythium aphanidermatum, Rhizoctonia solani* and *Fusarium oxysporum* was evaluated under *in vitro* conditions.

KEY WORDS : *Limnophila repens*, GC/MS, terpinen-4-ol , sabinene, antifungal activity.

INTRODUCTION

Limnophila (meaning pond-loving in latin) is an important genus of the family *Scrophulariaceae* comprising of 40 species of aquatic or semi-aquatic plants growing in marshes, riversides, forest paths or similar wet region. They are native to Africa, Asia, Australia and Pacific islands, in tropical to subtropical areas [1]. The paddy farmers treat them as serious weeds while they are widely used in traditional folk medications [2]. The plant under investigation *Limnophila repens*. Benth is an aquatic herb distributed mainly in tropical Asia (India, Bangladesh, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand etc) and Australia [1]. In the Indian native language *malayalam* it is

called 'manganari" which means 'smelling mango' indicating its pungent aroma reminiscent of tender mango fruits.

In ancient Indian medical texts of 'Ayurveda' *L.repens* is recommended as a substitute for *L.aromatica* which is described to be possessing antiseptic, appetizer, digestive, carminative, anthelmintic, anti-inflammatory, diuretic and febrifuge properties.[3],[4]. In Chineese medicine *L.aromatica* is used for the treatment of intoxication, body pains and for menstrual problems. [5]. The local medical people report *L.repens* as an efficient an anthelmintic drug and an ingredient for anti-inflammatory preparations .

Limnophila plants are aromatic herbs with pleasant smelling volatile oils. A good deal of research data is available on the composition and biological properties of *limnophila* essential oils . Essential oil from the dried areal parts of *L.geoffrayi* found to be comprised of d-pulgone (27%), perillaldehyde (19%) and limonene (9%) as the major components [6]. It showed good inhibitory activity on microorganisms present in contaminated cosmetic products. Volatile oils from the leaves and stems of *L. chinensis ssp. aromatica* collected from North American market was dominated by limonene (53%) and cis-4-caranone(12%) [7]. Yu Xuejian et. al reported estragole (21.94%) and trans-anethole (76.39%) as the major components in the volatile oil of *L.rugosa* [8].

Essential oil from *L.aromatica* collected from Bangladesh were rich in z-Ocimene (39%), terpinolene (17%) and camphor (13%) [9]. In another report δ-limonene and δ- perillaldehyde appeared as the major constituents of the oil which showed bactericidal and fungicidal activity [10]. Volatile oil from *L.indica* leaves was characterized by monoterpenes and long chain fatty acids [11]. α-Phellandrene (52.2%) and thymol (38.2%) dominated the oil from *L.conferta* which showed considerable anti-inflammatory, antifungal and anthelmintic activity [12].

Essential oil from *L. repens* was also mentioned in more than one occasion. The internet resource PROSEA (Plant Resources of South-East Asia) Foundation, Bogor, Indonesia reported that the essential oil from *L.repens* collected from south east Asia contained α-phellandrene (52.2%) and thymol (38.2%) as the major components. It is clear that *L.conferta* is treated as identical to *L. repens* here for this report as the major compounds in the oil from *L. conferta* were reported as α-phellandrene (52.2%) and thymol (38.2%) by Reddy et.al [12]. These botanical names are taken as 'synonymous' by some taxonomists. δ-Limonene was reported as the major component in the volatile oil in another study [13] resembling the essential oil from *L. aromatica* [10]. As these plants are similar in many botanical appearances, occupying the same ecological regions and possessing the same pungent aroma minor errors in the plant selection can be suspected. So we decided to revisit the essential oils from *L. repens* for its chemical

composition as well as for antifungal potentials. Olfactory evaluation of the volatile oil was also done which is for the first time in the case of any plant in the genus *Limnophila*.

MATERIAL AND METHODS

Plant Collection : The fresh plant material was collected from paddy fields of Kannur district, Kerala, India. It was identified by Dr. A.K. Pradeep, Department of Botany, University of Calicut and a specimen voucher is deposited in the specially maintained Herbarium of Chemistry department of University of Calicut.

Extraction of Oil : The aerial parts of the plant (500 g) was washed and ground into a paste and subjected to steam distillation for 3 hours. The oil was extracted by diethyl ether from the distillates. The ether extract was dried using anhydrous sodium sulphate and the ether evaporated. The pure oil was stored below 4^{o}C until analyzed.

Gas chromatography/mass spectrometry/olfactometry: GC/MS/O was carried out using an Agilent 6890 gas chromatograph, fitted with a HP-5 (5 % diphenyl polysiloxane) capillary column (50 m x 0.32 mm x 0.52 μm), with He carrier gas, initial head pressure 15.0 psi (2.0 mL/min) constant flow mode. The column effluent was split between an Agilent 5975N inert MSD spectrometer and an in-house odour detection port *via* a Capillary Flow Technology splitter plate with pressure set to 3.8psi. The injector and odour port transfer line temperatures were held constant at 230°C and 250°C, respectively. Injection of 1μl at 500ng/μl dilution in splitless mode with oven program: 35°C (3min), 15°C/min ramp to 50°C then 5°C/min ramp to 280°C(held 10min). Data was acquired and processed using MSD ChemStation (Rev. D.02.00.275). The odour assessments and description were carried out by experienced perfumers.

Gas chromatography/mass spectrometry: GC/MS was carried out using the same system but optimised for resolution with a linear temperature ramp of 2°C/min and calibrated for Retention Indices using C7-C28 n-alkanes. The inert MSD was operated with source temp 230°C, quad temp 150°C and ionization voltage 70 eV. Target spectra were acquired using the s.tune parameters and compared against in-house and commercial libraries from which identifications were assigned on the basis of both spectral match and retention data [14].

Gas chromatography/flame ionization detection: GC/FID analysis for quantisation was carried out using an Agilent 6890 gas chromatograph, fitted with an Ultra 2 (5 % diphenylpolysiloxane) capillary column (50 m x 0.2 mm x 0.33 μm), split injection (50:1) with He carrier gas (1.2 mL/min). The oven was programmed from 50–280°C

(held for 6 min) at 2°C/min. The injector and detector temperatures were held constant at 230°C and 300°C, respectively. Data was acquired and processed using HP ChemStation software (Rev. A.10.02 [1757]). Quantitative data was obtained from relative peak area (%RPA) without the use of response factors.

Analysis of Antifungal Activity by in vitro bioassay

A definite amount of each oil was accurately weighed out in to a clean standard flask and made up to 100 ml using diethyl ether. Various concentrations of test solutions were prepared in PDA (Potato Dextrose Agar) medium to obtain concentrations such as 5.0ml/100ml, 10.0ml/100ml, 15.0ml/100ml and 20ml/100ml of the ether solution before autoclaving the medium. It was also tested that, whether the activities were lost during autoclaving, and it was confirmed that, the oil is thermo-stable. In addition, two controls were (control with respective concentrations of diethyl ether as control and without any test solution or diethyl ether as absolute control) also maintained. For each treatment, three replications were maintained.

RESULTS AND DISCUSSION

Composition : The hydrodistillation of the plant *L.repens* gave a pale green coloured oil with a yield of about 0.03% of the fresh weight sample. The oil was slightly pungent, but pleasant smelling. From the GC/MS analysis 33 compounds consisting of 91.09% of the oil were identified (Table 1). The oil was dominated by 19 monoterpenes followed by 7 aliphatic compounds and 5 sesquiterpenes. Monoterpenes consisted of 12 hydrocarbons and 6 alcohols .

It is the presence of ascaridole the rarely occurring natural organic peroxide and the only natural terpene peroxide to be specially mentioned. It is the first report of this compound from this plant and the whole genus *limnophila*. Ascaridole was the first, and for a long time only discovered naturally occurring organic peroxide isolated from the oil of Mexican tea plant as the major component [15] . For many years it was a major remedy against intestinal parasites in humans and other household animals [16]. It is interesting to note that the Mexican tea plant itself is traditionally used in tonic drinks and infusions to expel intestinal parasites in folk medicine practiced in North and South Americas, China and Turkey [17]. Biogenesis of ascaridole was shown to be starting from α-terpinene and catalyzed by a soluble iodide peroxidase [18] in different plants like *Chenopodium ambrosioides.* α-Terpinene was present in our sample while the presence of peroxidase enzyme is yet to be proved. Even though present in low

amount in the essential oil, it will be none other than ascaridole contributing to the traditional anthelmintic property to *L.repens* as in the case of 'worm seed' alias Mexican Tea plant.

The oil consisted of terpinen-4-ol (19.33%) as the major component followed by sabinene(18.34%) , terpinolene(7.29%), oct-1-en-3-ol(7.2%), trans-β-farnesene (6.78%) and γ-terpinene (6.39%). These compounds had been previously reported from the *limnophila* but it is the first time as major components of the essential oil from any plant of the genus. The presence of valencene, an aroma component of citrus fruits and the precursor of nootkatone was another peculiarity of this oil. The major components of the earlier reports of oil from so called *L.repens* α- phellandrene, limonene and p-cymene were also present, but in very low concentration. These marked differences cannot be attributed to regional variations only but make it clear that *L.repens* is a different chemotype of the genus compared to *L. conferta* and *L. aromatica*.

Olfactory Evaluation: The odour impressions and odour threshold values [19] of the constituents of the essential oil are given in table 1. *Odor threshold value (OTV)* (or *aroma threshold value (ATV),*) is defined as the most minimal concentration of a substance that can be detected by a human nose. This value varies from substance to substance and expressed as a concentration in water or concentration in air usually in ppb. Generally the odour impressions corresponding to compounds with low OTV dominates in human perception . So the impressions of α-pinene (pine, turpentine), Oct-1-en-3-ol (mushroom), p-Cymene (solvent, gasoline,), Linalool (Fresh, floral) and that of nootkatone (grapefruit) are to be expected as the major notes in the oil. These are followed by that of limonene (citrus, mint), myrcene (spicy, green mango), eugenol (spicy, clove) and that of trans- ocimene (herbal). Another compound terpinolene was also reported for its typical green mango like aroma [20] ; but the corresponding note may not be theoretically strong enough due to the high OTV . However the familiarities with tender mangoes in the regions make the recognition of the specific mango-note first and above all other impressions to name the plant as ‘mangannaari’ (mango-smelling). The flavor of mango ginger (*Curcuma amada* Roxb.) another herb with mango-like odour was attributed in some extent to *cis*-ocimene [21] which was also present in this oil enhancing the specific impressions.

Antifungal studies : The antifungal *in vitro* study (Table 2) revealed that the oil possess considerable antifungal properties. It showed good growth inhibitory effect on *P. aphanidermatum*(maximum of 94% for 24 hrs) and

F.oxysporum (maximum of 72%) but less activity on *R.solani*. Even at low concentration of 05 ml/100ml the microbial growth was sufficiently inhibited and increase in concentration showed a positive drift in the activity. Fungicidal activity of the oil can be attributed to the monoterpenes terpinen-4-ol, sabinene, terpinolene and γ-terpinene which are its major components. Terpinen-4-ol had been several times reported for its similar biological properties [22], [23]. Antifungal activity terpinolene [24] and γ-terpinene [25] had also been reported. Generally major components are responsible for the biological activity of essential oils, but sometimes the minor components also play major role making the whole oil more active than the combination of major components in a synergistic effect [26]. It is to be mentioned that essential oil from *L.repens* collected from south east Asia had been earlier reported to be sensitive against *Aspergillus niger*, *Candida albicans* and *Rhizopus nigricans* [10].

CONCLUSIONS

It can be concluded that the volatile oil from the aquatic weed *L.repens* consists mainly of monoterpenes with vital medicinal properties. The individual components possess specific olfactoric impressions imparting characteristic tender mango like odour to the plant. Fungal infection is an ever challenging threat to the agro-life in the same sense as natural calamities while many of the synthetic antifungal agents are harmful to the ecosystem. It is better news for the agro-men that the plants which are usually treated as unwanted weeds like *L.repens* can be utilized for fungicidal preparations aimed at notorious plant pathogens.

Acknowledgements

Mohamed Shafi, P. is thankful to UGC, New Delhi (SAP Programme) for financial assistance and Mohanakrishnan, M. is thankful to UGC, New Delhi for his fellowship under FIP. The authors are grateful to Dr Robin Clery, Givaudan AG, Dübenodorf for assistance with GCMS and olfactometry.

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Table 1. GC/ MS Analysis with FID quantification of *L.repens* oil

No	RI	RI*	Compound	Percentage	Odour Impression	OTV in water (ppb) [19]
1	852.7	852	cis- 3-Hexenol	0.27	Faint green	70
2	863.1	863	trans- 2-Hexenol	0.36	Green, leafy	400
3	865.4	865	Hexanol	0.26	resinous, green	2500
4	927.9	928	α-Thujene	1.09	wood, green, herbal	-
5	935.4	935	α- Pinene	0.46	pine, turpentine	6
6	975.8	975	Sabinene	18.34	pepper, wood	37
7	978.8	978	Oct-1-en-3-ol	7.2	Weak, mushroom	1.2
8	985.8	987	Octan-3-one	0.19	Herbal, resinous	28
9	991.4	993	Myrcene	2.64	spicy, green mango	13-15
10	994.8	995	Octan-3-ol	0.73	faint mushroom	110-130
11	1006.6	1007	α- Phellandrene	0.38	turpentine, mint,	200
12	1018.7	1018	α- Terpinene	2.76	lemon	260
13	1026.3	1027	p-Cymene	0.78	solvent, gasoline,	6.2
14	1030.8	1031	Limonene	1.4	citrus, mint	10
15	1031.6	1032	β- Phellandrene	1	mint, terpentine	200
16	1048.7	1048	trans-Ocimene	0.3	sweet, herb	34
17	1061.1	1060	γ- Terpinene	6.39	gasoline, turpentine	260
18	1091.3	1090	Terpinolene	7.29	slightly green mango, sour	200

Table 1.....

19	1100.2	1101	Linalool	0.6	Fresh, floral	6
20	1124.3	1125	cis-p-Menth-2-en-1-ol	0.82	Herbal	N.A
21	1142.2	1142	trans-p-Menth-2-en-1-ol	0.64	green hay	N.A
22	1170.4	1172	Borneol	0.57	Camphorous	140
23	1182.8	1181	Terpinen-4-ol	19.33	Medicinal,	1200
24	1194	1194	α-Terpineol	0.9	oil, anise, mint	330
25	1290.5	1291	Bornyl acetate	0.27	camphoreous,	75
26	1310.3	1308	Ascaridole	0.32	pungent	NA
27	1361.1	1363	Eugenol	0.5	Spicy, clove	6-30
28	1430.6	1431	Caryophyllene	4.41	Woody, spicy	64
29	1460	1460	trans-β-Farnesene	6.78	Woody, sweet	NA
30	1464.7	1465	α-Humulene	1.26	woody	NA
31	1503.5	1505	Valencene	1.15	Green, oily	NA
32	1820.4	1822	Nootkatone	1.12	grapefruit	0.8-1
33	1840.7	1842	Neophytadiene	0.58	Not specific	NA
Total				91.09		

RI- Measured Retention Index RI*- DB-5 ref.

Table 2. Effect of *L.repens oil* on *P. aphanidermatum, R. solani* and *F. oxysporum – in vitro* – (Percentage Inhibition)

On *Pythium*			
Concentration	After 24 hrs	After 48hrs	After 72 hrs
05.0ml/ 100ml	44.79	43.46	45.43
10.0ml/100ml	46.28	44.04	36.25
15.0ml/100ml	52.28	71.43	62.93
20ml/100ml	94.09	72.04	69.18
On *Rhizoctania*			
Concentration	After 24 hrs	After 48hrs	After 72 hrs
05.0ml/ 100ml	57.58	14.44	0.00
10.0ml/100ml	37.33	0.00	0.00
15.0ml/100ml	72.16	40.38	0.00
20ml/100ml	59.48	24.82	0.00
On *Fusarium*			
Concentration	After 24 hrs	After 48hrs	After 72 hrs
05.0ml/ 100ml	0	6.70	11.72
10.0ml/100ml	0	0	0
15.0ml/100ml	59.13	30.00	15.03
20ml/100ml	72.75	35.00	21.66