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GC-MS Analysis, Antibacterial and Antifungal activity of essential oil of *Plectranthus rugosus* from Kashmir, India.

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Abstract: This work was carried out to evaluate chemical composition, antibacterial and antifungal activity of *Plectranthus rugosus* essential oil. The oil was extracted by hydro distillation which was analyzed through GC-MS. The antibacterial and antifungal activity was evaluated by Agar well diffusion method and Minimum inhibitory concentration (MIC) was evaluated by Agar dilution method. Caryophyllene, Germacrene D, alpha-pinene and beta-cymene were the major constituents present in the oil. The oil showed significant antibacterial and antifungal activity.

Key words: *Plectranthus rugosus*; GC-MS; Antibacterial and Antifungal Activity.

INTRODUCTION

The genus *Plectranthus* is a member of the family umbelliferae. It is comprised of eighty species, which are widely distributed in tropical and sub-tropical regions of Asia, Africa, Australia and Polynesia. Twenty-one species of *Plectranthus* are found in India. Out of these *P. gerandianus*, *P. Striatus* and *P. rugosus* grow wild in the state of Jammu and Kashmir (Hooker, J.D). In Kashmir, the species *P.rugosus* is widely distributed at an altitude of 3000 to 8000 ft. It is a tall shruby plant, also found in Bhutan and Afghanistan. The plant is erect, possess small petioled, ovate or elliptic obtuse leaves, Cymes are auxiliary laxfid and panicled. Corolla tube very short, lips very large, calyx hairy, teeth subequal acute, nutlets oblong. *P. rugosus* flowers from July to September and the seeds ripen from August to October. The plant is used in traditional medical practices in tooth ache and is claimed to be effective as an antiseptic, a hypoglycaemic, an anti-diarrheal and a bronchodilator (Ajmal SM, Mohammad S, Zahid K, Bakht Z; 2012 Sher Z, Khan Z, Hussain F; 2011). A topical administration of fresh leaf extract is used to treat scabies for its immediate effect, while 1-2drops of this extract are used to treat earache (Sabeen M, Ahmad SS, 2009). An extract of leaves is also used to treat hypertension, fevers, rheumatism and toothache. Branches are used for making dusters (Khan SW, Khatoon S; 2007, Akhtar N, Rashid A, Murad W, Bergmeier E; 2013). Previous phytochemical investigation of the plant revealed the presence of steroids, flavonoids, terpenoids, saponin, tannins, cardiac glycosides, coumarins, reducing sugars and β-cyanin among the methanol soluble extractable constituents. From the plant, some diterpenoids, i.e. rugosinin, effusanin-A, effusanin-B, effusanin-E, lasiokaurin and oridonin, have been isolated. Moreover, the analysis of volatile oil fractions from leaves and inflorescence indicated presence of sesquiterpene hydrocarbons, including β-caryophyllene, germacrene-D and α-humulene as the major constituents (Padalia RC, Verma RS; 2011). Furthermore, plant extracts and fractions by different solvents exhibited antifungal (Rauf A, Khan A, Rasool S, Shah ZA, Saleem M; 2012), antibacterial, phytotoxic (Rauf A, Muhammad N, Khan A, Uddin N, Atif M; 2012) and antioxidant activities (Rauf A, Uddin G, Ali M, Muhammad N, Gul S; 2013).

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MATERIALS AND METHODS

Plant material The leaves of *Plectranthus rugosus* were collected from Pahalgam region of Kashmir valley in juiy-2013. The plant sample was identified and authenticated by Dr.A.R. Naqshi, taxonomist under specimen vocher number-KUIK01. The specimen has been deposited in the herbarium of pharmaconosy and phytochemistry laboratory, Department of Pharmaceutical Sciences, University of Kashmir.

Essential oil isolation

The chopped leaves of *P. rugosus* were used and the essential oil was obtained by hydrodistillation in a clavenger type apparatus as recommended by European Pharmacopeae. The yield of oil, as calculated on fresh weight basis (v/w), was 0.256%. The oil sample was dried over anhydrous sodium sulphate and stored in a sealed glass vial in a refrigerator at 4°C prior to analysis.

Chemical Composition

GC-MS Analysis: GC-MS analysis was carried on a Varian Gas Chromatograph series 3800 fitted with a VF-5ms fused silica capillary column (60mx0.26mm, film thickness 0.25µm) coupled with a 4000 series mass detector under the following conditions: injection volume 0.20µl with split ratio 1:60, helium as carrier gasat1.0ml/minconstant flow mode, injector temperature 230°C, oven temperature 60°C to 280°C at 3°C /min.

Antimicrobial assay

Microbial strains and culture media: Gram positive and Gram negative bacterial strains and the fungal strains were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigrah, India. The bacterial strains used were *Pseudomonas aeruginosa* MTCC 1688, *E. coli* MTCC 407, *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 96, *Proteus vulgaris* MTCC 426, *Klebsiella pneumonia* MTCC 19. The fungal strains used were *Saccharomyces cerevisiae* MTCC 1023, *Candida albicans* MTCC 6258, *Pencillium crysogenum* MTCC 1380, *Aspergillus fumigates* MTCC 9001. The Muller Hinton Agar and Sabouraud Dextrose Agar media were

used for the determination of antibacterial and antifungal activity respectively. The bacterial strains were grown on MHA plates and MHA slants at 37°C and later on refrigerated until further use. The fungal strains were grown on SDA plates and SDA slants at 28°C and maintained under refrigeration.

Antimicrobial activity and determination of Minimum inhibitory concentration (MIC)

Agar well diffusion assay: The antimicrobial susceptibility tests were carried out using the Agar well diffusion assay (Irith Wiegand *et al.,* 2008). The bacterial cultures were developed for 24 hours and fungal cultures were developed for 48 hours and later transferred into boiling tubes containing 20 ml of liquid MHA and 20 ml of SDA respectively. The contents of the tubes were transferred to petriplates. After 5 minutes of solidification of the agar, petriplates were punched in the form of wells. Later these wells were filled with different concentration of oils (10µl, 20µl, 30µl) for bacterial assay and (20µl, 30µl) for fungal assay. The incubation was carried out for 24hours at 37°C for bacteria and for fungi incubation period was 48 hours at 28°C. After the incubation period, the antimicrobial activity was evaluated by measuring the width of zone of inhibition. The aqueous solution of streptomycin sulphate (6µl) was used as positive control in case of antibacterial activity while as Amphotericin B (20µg /disc) was used as positive control for antifungal activity. However, DMSO was used as negative control.

Minimum inhibitory concentration (MIC) assay: The essential oil of *Plectranthus rugosus* displayed significant and broad spectrum antibacterial and antifungal activity against different bacteria and different fungi used. Minimum Inhibitory Concentration of oils was determined by Agar Dilution Method, recommended by Clinical Laboratory Standards Institute (CLSI) (Irith Weigand *et al.,* 2008). A series of two fold dilutions of the oils ranging from 0.2-25.6 mg/ml was prepared in MHA at 48°C and in SDA at 40°C for antibacterial and antifungal activity respectively. Plates were dried at room temperature for 30 minutes prior to spot inoculation with 3µl and 2µl aliquots of culture containing approximately 10^5cfu/ml and 10^3cfu/ml of each organism for antibacterial and antifungal activity respectively.

The bacterial plates were incubated at 37°C for 18 hours while the fungal plates were incubated at 28°C for 48 hours and were read visually and MIC was determined. Experiments were performed in triplicate. Inhibition of bacterial growth and fungal growth in the plates containing test oil was judged by comparison with growth in blank control plate. The MICs were determined as the lowest concentrations of oil inhibiting visible growth of each organism on the agar plate. The MIC for different strains of bacteria and fungi are presented in tables 3 and 5.

RESULTS AND DISCUSSION

The different essential oil constituents of the leaf of *Plectranthus rugosus* are shown in table-1, in order of their elution from RTX-5 column. GC-MS analysis led to the identification of 34 chemical constituents accounting for 100% of the total oil composition.

Table 1: Chemical composition of essential oil of *Plectranthus rugosus* by GC/MS

S.No.	Peak Name	RI (min)	Area	%age	Method of identification
1.	alpha-Thujene	13.576	19394	0.650	MS,RI
2.	alpha –Pinene	14.011	292524	9.802	MS,RI
3.	1-0CTEN-3-0L	16.492	118321	3.965	MS,RI
4.	beta –Pinene	16.968	103581	3.471	MS,RI
5.	alpha –Phellandrene	17.977	193703	6.491	MS,RI
6.	3-Carene	18.106	38637	1.295	MS,RI
7	beta cymene	18.971	262480	8.795	MS,RI
8.	Limonene	19.221	72035	2.414	MS,RI
9.	beta –Phellandrene	19.329	112944	3.784	MS,RI
10	beta -trans-Ocimene	20.057	36098	1.210	MS,RI
11	alpha Terpinene	20.765	34635	1.161	MS,RI
12.	Terpinolene	22.216	22663	0.759	MS,RI
13	Linalool	22.886	36927	1.237	MS,RI
14.	4-Terpineol	27.181	19668	0.659	MS,RI
15.	p-Cymen-8-ol	27.490	6192	0.207	MS,RI
16	alpha Terpineol	27.897	7261	0.243	MS,RI
17	2-Methyl-1-cyclohexen-1-yl) methanol	33.859	45000	1.508	MS,RI
18	Copaene	36.309	16258	0.545	MS,RI
19	beta –Bourbonene	36.701	12609	0.422	MS,RI.COI
20	Caryophyllene	38.295	316234	10.596	MS,RI.COI
21	alpha Caryophyllene	39.840	118211	3.961	MS,RI
22	Germacrene D	40.878	296258	9.927	MS,RI
23	alpha Muurolene	41.510	69403	2.326	MS,RI
24	6.alpha.-CADINA-4.9-DIENE, (-)-	42.158	37593	1.260	MS,RI
25	delta –Cadinene	42.296	133360	4.469	MS,RI
26	CalameneneI	42.478	51685	1.732	MS,RI
27	3,5-Diisopropenyl-1,1,2-trimethylcyclohe	43.241	13918	0.466	MS,RI
28	Spathulenol	44.788	16807	0.563	MS,RI
29	Caryophyllene oxide	45.066	99680	3.340	MS,RI
30	tau –Gurjunene	45.534	97879	3.280	MS,RI
31	tau –Muurolene	47.268	90421	3.030	MS,RI
32	CedreanoL, (-)-	47.26	29778	0.998	MS,RI
33	tau.-Muurolol	47.777	116044	3.888	MS,RI
34	beta –Bisabolene	48.774	46187	1.548	MS,RI
	Total identified percentage			**100.00 %**	
	Class composition				
	Monoterpene hydrocarbons			48.07%	
	Sesquiterpene hydrocarbons			51.93%	

MS -Mass Spectroscopy;
RI- Retention indices in elution order from RTX-5 columns,
COI-Co injection, Retention time identical to Authentic compounds
Results presented are the means of three replicative isolations %, relative percentage obtained from peak area.

Table 2: *In vitro* antibacterial activity of *Plectranthus rugosus* essential oil and reference antibiotic determined with agar well diffusion method

S.No.	Test Organisms	Conc. of essential oil in µl to determine zone of inhibition (diameter in mm)			Standard used (streptomycin in µl)
		10 µl	20µl	30µl	6µl
01	*Staphylococcus aureus* MTCC 96	16±0.57	17±0.57	18±0.57	28±0.57
02	*Bacillus Subtilis* MTCC 441	16±0.57	16.6±0.57	19±0.57	28±0.57
03	*Pseudomonas aeruginosa* MTCC 1688	17±0.57	19±0.57	22±0.57	27±0.57
04	*Klebsiella Pneumoniae* MTCC 19	17±0.57	19±0.57	20±0.57	23±0.57
05	*Proteus vulgaris* MTCC 426	18±0.57	20±0.57	22±0.57	29±0.57
06	*Escherchia coli* MTCC 443	13±0.33	16±0.57	18±0.57	27±0.57

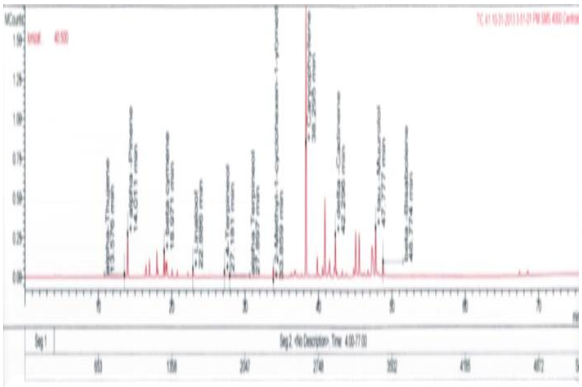

Figure 1: GC/MS Chromatogram of the essential oil of *Plectranthus rugosus leaf*

Antibacterial and Antifungal Activity

The *In vitro* antibacterial and antifungal activity of essential oil was qualitatively and quantitatively assessed by the presence or absence of Inhibition Zones, Zone diameters and Minimum inhibitory concentration (MIC) values. Results from antibacterial and antifungal activity by Agar well diffusion method are presented in tables 2 and 5. The essential oil of *Plectranthus rugosus* showed strong antimicrobial effects against different bacterial and fungal strains used for screening. The maximum Zone of Inhibition was measured in *P. vulgaris* (23mm) bacterial strain. The MIC of the oil ranged from 3.2 -12.8mg/ml. The Zone of Inhibition was lesser for *E. coli* (19mm) compared to other bacterial strains. In case of Antifungal activity, the maximum Zone of Inhibition was measured in *P. crysogenum* (30mm) fungal strain. The MIC of Oil ranged from 1.6 - 6.4mg/ml. The Zone of Inhibition was lesser for *A. fumigatus* (12mm) compared to other fungal strains.

Streptomycin sulphate (6µl) was taken as a positive control for antibacterial activity, while as Amphotercin B (20µg/disc) was taken as positive control for antifungal activity. However, no activity was observed with DMSO which acted as Negative control during the whole process.

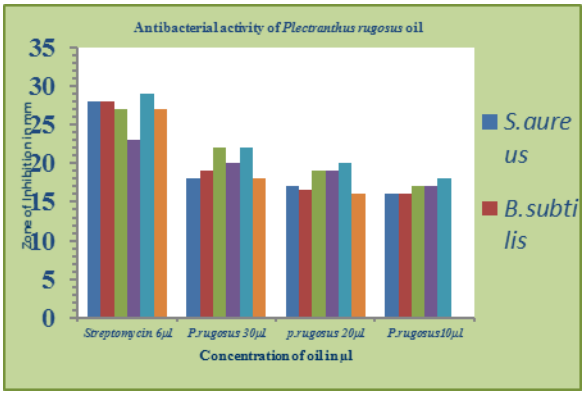

Figure 2: Graphical representation of zone of inhibition of the *Plectranthus rugosus* oil against different bacterial strains.

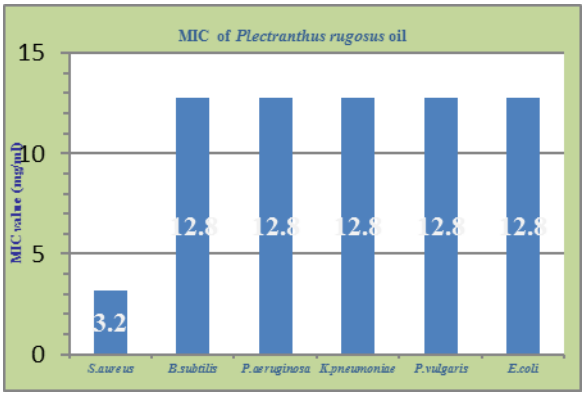

Figure 3: Graphical representation of Minimum inhibitory concentration (MIC) value of the *Plectranthus rugosus* oil against various bacterial strains.

Table 3: Minimum Inhibitory Concentration (MIC) of *Plectranthus rugosus* oil (mg/ml) against different bacterial strains

Oil name	Test micro organisms					
	***S. aureus* MTCC 96**	***B. Subtilis* MTCC 441**	***K. pneumoniae* MTCC 19**	***P. vulgaris* MTCC 426**	***P. aeruginosa* MTCC 1688**	***E. coli* MTCC 443**
Plectranthus rugosus	3.2mg/ml	12.82mg/ml	12.82mg/ml	12.82mg/ml	12.82mg/ml	12.82mg/ml

Table 4: *In vitro* antifungal activity of *Plectranthus rugosu*s essential oil and reference antibiotic determined with agar well diffusion method

S. No.	Test Organisms	Concentration of essential oil used in µl to determine zone of inhibition (diameter in mm)		Negative Control used (DMSO)	Standard used
		20µl	30µl	40 µl	Amphotericin B 20 µg /disc
01.	*Saccharomyces cerevisiae* MTCC 1023	14±0.57	15±0.57	0mm	11±0.57
02.	*Candida albicans MTCC 6258*	12.6±0.3	13.6±0.3	0mm	11±0.57
03.	*Pencillium Crysogenum* MTCC 1380	24±0.57	29±0.57	0mm	28±0.57
04.	*Aspergillus fumigatus* MTCC 9001	9.6±0.33	11.6±0.3	0mm	16±0.57

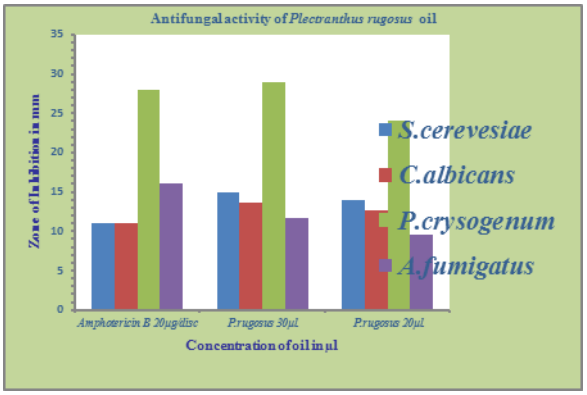

Figure 4: Graphical representation of zone of inhibition of the *Plectranthus rugosus* oil against different bacterial strains.

Table 5: Minimum inhibitory concentration (MIC) of the essential oil of *Plectranthus rugosus* (mg/ml) against different fungal strains:

Oil name	Fungal organisms			
	***S. cerevisiae* MTCC 1023**	***C. albicans* MTCC 6258**	***P. crysogenum* MTCC 1380**	***A. fumigatus* MTCC 9001**
Plectranthus rugosus	6.4mg/ml	6.4mg/ml	6.4mg/ml	1.6mg/ml

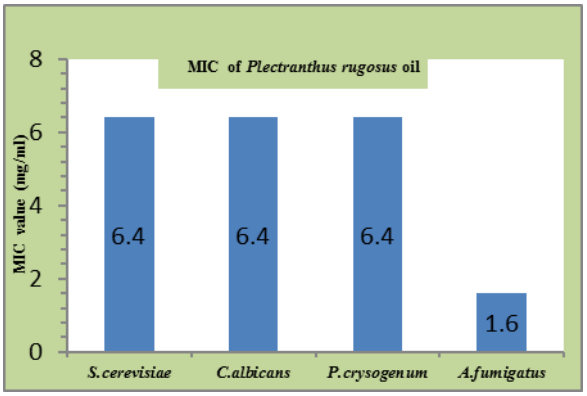

Figure 5: Graphical representation of Minimum inhibitory concentration (MIC) value of the *Plectranthus rugosus* oil against various fungal strains.

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