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Research Article

PHYTOCHEMICAL INVESTIGATION AND IN VITRO ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL FROM THE RHIZOME OF BERGENIA STRACHEYI (Hook.f. & Thomson) Engl.

Pawan Kumar¹, Mohammad S Javed^{1*}, A. K. Tewari², K. S. Bisht²

¹Department of Chemistry, Kumaun University, Nainital 263002, Uttarakhand, India

²Department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture & Technology, Pantnagar 263145, Uttarakhand, India

*Corresponding Author's E-mail address: msuhail12003@yahoo.co.in

ABSTRACT

The essential oil from the rhizome of *Bergenia stracheyi* (Hook.f. & Thomson) Engl. growing in the Himalayan region of Jammu & Kashmir is found rich in oxygenated sesquiterpenoids (43.06%) and higher oxygenated hydrocarbons (29.83%). Sesquiterpenoids (0.46%) and monoterpenoids (0.24%) were almost absent in the oil. Among oxygenated sesquiterpenoids Cadinene <14-hydroxy- δ -> (38.36%) and Khusinol (2.06%) were the main components. The higher hydrocarbons and their derivatives include n-Hexadecanoic acid (10.60%), *cis*-9-hexadecenoic acid (4.46%), Linoleic acid (2.85%), Octacosane (2.52%), Cetanol (2.26%), Oleic acid (2.14%), Nonacosane (1.86%), n-Pentacosane (1.66%) and Untriacontane (1.48%). The oxygenated monoterpenoids include α -Terpineol (1.10%) as main constituent. The oil also contains m-xylene (11.27%) as the major lower hydrocarbon. The decoction of rhizome from *Bergenia stracheyi* are used in folk lore medicines in Jammu & Kashmir. The oil from the rhizome has shown moderate antifungal activity with 100% mycelia growth inhibition against *S. sclerotiorum* and *A. tenuis* at a concentration of 2000 μ g/ml and 3000 μ g/ml respectively. However *C. graminicola* and *F. oxysporum* were found less susceptible to this oil. The IC₅₀ values showed a range from 802.7 μ g/ml to 1615.4 μ g/ml as compared to standard fungicides with IC₅₀ values ranging from 36.8 μ g/ml - 78.6 μ g/ml. The spore germination inhibition test has revealed this oil as a moderate inhibitor with IC₅₀ values 899.6 μ g/ml, 545.2 μ g/ml and 2014.7 μ g/ml for *A. tenuis*, *C. graminicola* and *F. oxysporum* spores respectively.

Keywords: Saxifragaceae, Alternaria, essential oil, fungicide, terpenoids.

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1. INTRODUCTION

The Genus *Bergenia* belongs to family Saxifragaceae represented by 80 genera and 1200 species with 32 genera in India, distributed between 2600m - 4600m from Kashmir to Kumaon Himalaya¹. Both leaves and rhizome of *Bergenia* possess medicinal value. Three flavonol glycosides namely Quercetin-3-O- α -L-rhamnoside, Kaempferol-3-O- α -L-rhamnoside and Kaempferol-3-O-rhamnosyl (1-6) glycoside

have been reported from leaf extract of *B. stracheyi*². The whole plant extract from *B. stracheyi* has shown the presence of amino acids, proteins, carbohydrates, glycosides, phenolics, steroids and terpenoids and the extract has radical scavenging activity³. The methanolic extract from *B. ciliate* rhizome has phenolics (5.78%), tannins along with flavonoids and gallic acid as major compounds, the extract scavenge

DPPH (1,1-diphenyl-2-picrylhydrazyl) and superoxide radicals⁴. Rhizome from *Bergenia* species is used in the ayurvedic formulation to dissolve kidney and bladder stones, leucorrhea, piles and pulmonary infections⁵. The methanolic roots extract of *B. ciliate* at highest concentration 10mg/ml has in-vitro inhibitory effect on calcium oxalate monohydrate crystal's nucleation and aggregation⁶. The leaf and root extract of *B. ciliate* has shown marked antifungal activity⁷. *Bergenia* species are used to get-rid of kidney and gall bladder stone and toxic waste products which accumulate in the alimentary canal and urinary system^{8,9}. Juice of the rhizome is used in case of in cases of asthma, urinary trouble and hemorrhoids¹⁰. The rhizome of *Bergenia stracheyi* is used as a folk medicine for its antiscorbutic, astringent, diuretic, febrifuge and ophthalmic properties¹¹. Our work reveals the photochemical composition and anti-fungal activity of rhizome oil from *Bergenia stracheyi* growing in the Himalayan region of Jammu & Kashmir against five Phytopathogenic fungi.

2. MATERIAL AND METHODS

2.1. Plant Material

The fresh plant material (rhizomes of *Bergenia stracheyi*) was collected from high altitudes of Bhallessa (Doda), Jammu and Kashmir (India), at an elevation of 2900m in the month of June 2014 when the plant was in its flowering stage. The rhizomes were washed with cold water and cleaned off dead skin and debris and were used for extraction of oil.

The preliminary plant identification was done by Prof. P. C. Pandey, Botany Department, Kumaun University, Nainital. The botanical identity was further confirmed by Botanical Survey of India, Dehradun, Voucher specimen [*Bergenia stracheyi* (Hook.f. & Thomson) Engl., Acc. No. 115214] where the herbarium specimen has been deposited.

2.2. Isolation of the essential oil

The essential oil was obtained by steam distillation of fresh plant material (4 kg rhizome) using a copper still fitted with spiral glass condenser. The distillate was saturated with NaCl and extracted with hexane. The hexane extract was dried using anhydrous sodium sulphate (Na₂SO₄) and the solvent was removed with a Rotovap at aspirator pressure and 36°C temperature to yield 2.1g of the oil (0.05% by weight). Residual oil was stored at 4°C till further analysis. All chemicals and reagents used were of analytical grade. Hexane, ether, anhydrous sodium sulphate (Na₂SO₄), and Dimethyl Sulphoxide (DMSO) were obtained from Merk, Mumbai, India where as potato dextrose agar (PDA), Potato dextrose broth (PDB) were obtained from Himedia, India.

2.3. Analysis of essential oil

2.3.1. Gas chromatography (GC-FID) and Gas chromatography-mass spectrometry (GC-MS) analysis

The analysis of the oil was done by using a gas chromatograph (Shimadzu GC QP 2010) equipped with RTx-5 MS capillary column, 1009701 (30.0m x 0.25mm, film thickness: 0.25µm). The oven temperature (50°C-280°C) was

programmed at 50°C for first 2 minutes, then 3°C/minutes to 200°C and then 10°C/minute to 280°C, after which it was maintained isothermally at 280°C for 8 min. N₂ was used as the carrier gas at 113.0mL/min. The injector temperature was 250°C, detector temperature 260°C and the injection volume 0.5µL using a 10% solution of the oil in n-hexane. The GC-MS analysis was carried out with GC-MS QP 2010 (Shimadzu) fitted with RTx-5 MS capillary column, 1009701 (30.0m x 0.25mm, film thickness: 0.25µm). The oven temperature (50°C-280°C) was programmed at 50°C for first 2 minutes, then 3°C/min. to 200°C and then 10°C/min. to 280°C. After which it was maintained isothermally at 280°C for 8 min. N₂ was used as the carrier gas. The injection volume 0.5µL and split ratio was 1:90. The mass spectra were taken at 70eV. The percentage by peak area normalization was taken to express the relative percentage of the oil constituents.

2.3.2. Compound identification

Identification of different constituents of the essential oil was done by comparing their Retention Indices (RI), in relation to a series of n-alkanes (C₆-C₃₃) indices on the RTx-5 MS capillary column, either with those of published data¹² or with authentic samples which were further supported by NIST08.LIB and WILEY8.LIB mass spectral library searches. The results are presented in table 1.

2.4. Plant pathogenic fungi

The foliage born and soil born fungi (table 2) were obtained from the Department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture & Technology, Pantnagar, India. The pure culture of these pathogenic fungal species were maintained on Potato Dextrose Agar (PDA) and stored at temperature below 4°C for further bioassay.

2.5. Preparation of pure culture and spore suspension

The Pathogenic fungi namely *Sclerotinia sclerotiorum*, *Alternaria tenuis*, *Colletotrichum graminicola*, *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *ciceris* were cultured on Potato Dextrose Agar (PDA) medium in sterilized Petri dishes (80mm in diameter). The 8 days old cultures of respective pathogens were used for harvesting spores in 10mL autoclaved distilled water using an inoculation loop. To obtain homogenous spore suspension, the suspension was centrifuged (Megafuge 1.0, Heracus Sepatech, Germany) at 1800rpm for 5 minutes. This suspension was serially diluted up to 10⁻² dilution to obtain countable spores (about 250-500 spores) on center large square of hemocytometer (B.S 748, I.S 10269, Rohem, India). The spores of each fungus were counted with hemocytometer and concentration (spores/ml) (table 2) in each plate was calculated using formula

$$\text{Spores per ml} = n \times 25 \times 10^4$$

Where n is the average no. of spores in Medium Square (0.04 mm²) of centered big square.

Serial concentrations of the oil were prepared by dissolving required amount of oil in 10% DMSO.

Table 1: Phytochemical composition of essential oil from the rhizome of *B. stracheyi*

S. No	Compound	% in the oil	Method of identification	
			RI ^a	others
1.	Santene	0.74	887	c, d
2.	n- Nonane	0.55	899	c, d
3.	m-xylene	11.27	907	b, c, d
4.	Menthene <-3-p->	0.09	987	c, d
5.	Mesitylene	0.11	995	c, d
6.	n- Decane	0.15	999	c, d
7.	trans-Limonene oxide	0.34	1090	c, d
8.	n-Nonanone	0.23	1094	c, d
9.	n-Nonanal	0.15	1106	c, d
10.	Camphor	0.69	1149	c, d
11.	Pinocarvone	0.09	1164	c, d
12.	Borneol	0.20	1172	c, d
13.	Umbellulone	0.26	1177	c, d
14.	Terpinen-4-ol	0.78	1181	c, d
15.	α -Terpineol	1.10	1194	b, c, d
16.	α -Copaene	0.17	1364	c, d
17.	δ -Cadinene	0.29	1528	c, d
18.	β -Caryophyllene epoxide	0.51	1583	c, d
19.	Globulol	0.78	1590	c, d
20.	γ -Eudesmol	0.42	1639	c, d
21.	Khusinol	2.06	1662	b, c, d
22.	Acorenone B	0.68	1698	c, d
23.	β -Acoradienol	2.06	1769	c, d
24.	Cadinene <14-hydroxy-δ->	38.36	1813	b, c, d
25.	Cetanol	2.26	1875	c, d
26.	cis-9-hexadecenoic acid	4.46	1959	b, c, d
27.	n-Hexadecanoic acid	10.60	1972	b, c, d
28.	Linoleic acid	2.85	2147	b, c, d
29.	Oleic acid	2.14	2153	b, c, d
30.	n-Pentacosane	1.66	2499	c, d
31.	Octacosane	2.52	2800	b, c, d
32.	Nonacosane	1.86	2895	c, d
33.	Untriacontane	1.48	3093	c, d
Sesquiterpenoids		0.46		
Oxygenated sesquiterpenoids		43.06		
Monoterpenoids		12.91		
Oxygenated monoterpenoids		3.84		
Higher hydrocarbons		29.83		
Total identified		90.10		

^a Retention index (RI) relative to homologous series of n-alkane (C6 – C32) on Rtx-5MS Capillary column.^b Compound checked by authentic standards compounds.^c Retention index (RI).^d MS, NIST and WILEY libraries spectra and the literature.**Table 2: Pathogenic fungi used for antifungal activity**

Pathogen	Host	Spores/ml
<i>Sclerotinia sclerotiorum</i>	Mustard	NA
<i>Alternaria tenuis</i>	Brinjal	3.4×10^6
<i>Colletotrichum graminicola</i>	Sorghum	6.3×10^6
<i>Rhizoctonia solani</i>	Rice	NA
<i>Fusarium oxysporum</i>	Chick pea	4.2×10^6

NA = Not appeared

2.6. In Vitro antifungal assay

Poisoned food technique¹³, using Potato Dextrose Agar (PDA) as medium, was used to check the antifungal activity of the oil against test fungi. The different concentrations of the essential oil were prepared by dissolving the appropriate amount of the oil in 10% DMSO and distilled water and then added into 20ml PDA to obtain desired concentration¹⁴. Mycelia plugs (2mm in diameter) from the edges of each culture were placed in the centre of each PDA plate (80mm). The control sets were prepared using equal amounts of 10% DMSO only and no oil. The prepared plates were inoculated aseptically with assay discs of the test fungus and were incubated at 25±2°C for 3-8 days until the growth in the control plates reached the periphery of the each plate. Growth inhibition of each fungal strain was calculated as the percentage inhibition of radial growth relative to control, using formula

$$\% \text{ mycelia inhibition} = \frac{C-T}{C} \times 100$$

Where C- is the radial growth in control plate and T- is the radial growth in the test plate.

The plates were used in triplicate for each treatment¹⁵. IC₅₀ (concentration that produces a 50% inhibitory effect) values were graphically obtained from the dose response curves based on measurements at six different concentrations.

2.7. Determination of minimum inhibitory concentration (MIC)

The MIC of the oil was determined by agar dilution method¹⁶. The oil sample was dissolved in 10% DMSO. A 10µl spore suspension (approx 10⁶ spores/ml) of each fungal strain was inoculated in the test tube in PDB medium and incubated for 4-8 days at 25±2°C. The control tubes containing PDB medium were inoculated only with fungal suspension. Where MIC is the minimum concentration of the oil in µg/ml at which no visible growth was observed.

2.8. Spore germination assay

Spore germination assay¹⁷ for *A. tenuis*, *C. graminicola* and *F. oxysporum f.sp. ciceris* with some modifications was done at different concentrations of oil. Aliquots of 30µl of essential oil solutions at different concentration (250-2000 µg/ml) were mixed with 30µl 5% dextrose solution and 40µl of the spore suspension (approx 10⁶ spores/ml) in cavity slide which were incubated in a moist chamber at 25±2°C for 24 hours. Each slide was then fixed in Lactophenol-cotton blue and observed under microscope for spore germination. The spores that produced germ tubes were enumerated and percentage of spore germination was calculated in comparison with control assay. Each assay was performed in triplicate and the results were obtained as average of the three results. The control having only 10% DMSO was tested separately for spore germination of different fungi. The percent spore germination was calculated by using formula

$$\% \text{ spore germination} = \frac{Ng}{Nt} \times 100$$

Where Ng is the number of spores germinated and Nt is the total number of spores examined.

2.9. Statistical Analysis

For all tests, the mean values and standard deviations were calculated. The data were analyzed using SPSS 16.0 statistical software. The one-way analysis of variance (ANOVA) was applied for calculating results. The means were compared by Duncan tests at a level of significance of P < 0.05.

3. RESULT AND DISCUSSIONS

3.1 Results

3.1.1 Composition of essential oil

The steam-distillation of fresh plant material (4kg rhizome) yielded 2.1g of colorless (transparent) oil (0.05% by weight) with pungent smell. The oil sample was analyzed by GC and GC-MS and the components were identified on the basis of their RI values, co-injection with the available authentic sample and by comparing their mass spectra with those reported in literature¹². In all 33 compounds were identified which constitutes about 90.10% of the oil. Detailed composition of the oil is presented in the **table 1**. The oil was found rich in oxygenated sesquiterpenoids (43.06%) and higher oxygenated hydrocarbons (29.83%). Sesquiterpenoids (0.46%) and monoterpenoids (0.24%) were almost absent in the oil. Among oxygenated sesquiterpenes Cadinene <14-hydroxy-δ-> (38.36%) and Khusinol (2.06%) were the main components. The higher hydrocarbons include n-Hexadecanoic acid (10.60%), *cis*-9-hexadecenoic acid (4.46%), Linoleic acid (2.85%), Octacosane (2.52%), Cetanol (2.26%), Oleic acid (2.14%), Nonacosane (1.86%), n-Pentacosane (1.66%) and Untriacontane (1.48%). The oxygenated monoterpenoids include α-Terpineol (1.10%) as main constituents. The oil also contains m-xylene (11.27%) as the major lower hydrocarbon and some trace compounds as other portion of the oil having less than 1% area.

3.1.2. In vitro antifungal activity

After an incubation period of 4 days for *S. sclerotiorum*, *R. solani* and 8 days for *A. tenuis*, *C. graminicola*, and *F. oxysporum f.sp. ciceris* at 25±2°C the effect of different concentrations of the oil from *B. stracheyi* are summarized in **table 3, Fig.1**. The oil inhibits the growth of mycelia strains in a dose dependent manner. The essential oil showed a varying effect at different concentrations. The oil was found effective against all the pathogenic test fungi. The inhibitory effect of the oil varied from 6.00% - 100% (**table 3, Fig. 1**). The oil of *B. stracheyi* completely inhibited the mycelial growth of *S. sclerotiorum* and *A. tenuis* at a concentration of 2000µg/ml and 3000µg/ml respectively. *C. graminicola*, *R. solani* and *F. oxysporum* are found less susceptible to this oil. The IC₅₀ and MIC values of the oil from *B. stracheyi* in comparison to fungicide (standard) showed moderate effect against *S. sclerotiorum* and *R. solani* (**table 4**).

Table 3: % mycelia growth inhibition^a by essential oil from *B. stracheyi*

Conc. µg/ml → Pathogenic fungi ↓	100 µg/ml	250 µg/ml	500 µg/ml	1000 µg/ml	2000 µg/ml	3000 µg/ml
<i>S. sclerotiorum</i>	00.00±0.00	6.25±0.03	10.00±0.20	20.00±0.50	100.00±0.00	100.00±0.00
<i>A. tenuis</i>	21.66±0.01	28.33±0.03	38.33±0.50	50.00±0.08	91.66±0.30	100.00±0.00
<i>C. graminicola</i>	12.50±0.90	15.00±0.10	22.50±0.20	37.50±0.06	56.25±0.90	87.50±0.20
<i>R. solani</i>	41.25±0.60	43.75±0.00	48.75±0.02	51.25±0.80	55.56±0.70	58.43±0.30
<i>F. oxysporum</i>	23.75±0.04	31.25±0.04	37.50±0.70	40.00±0.90	45.90±0.00	47.80±0.63

^a values within columns are given as mean ± S.D of three experiments.

Table 4: IC₅₀ and MIC values of essential oil and fungicides (positive control) against test pathogens

Pathogenic Fungi	Essential oil		Fungicide (positive control)			
	IC ₅₀ ^a	MIC ^b	Carbendazim		Mancozeb	
			IC ₅₀ ^a	MIC ^b	IC ₅₀ ^a	MIC ^b
<i>S. sclerotiorum</i>	1224.9	2000	36.8	500	NA	NA
<i>A. tenuis</i>	981.7	300	37.3	NA	36.4	500
<i>C. graminicola</i>	1615.4	NA	NA	NA	78.6	750
<i>R. solani</i>	802.7	NA	NA	500	NA	NA
<i>F. oxysporum</i>	1528.75	NA	128.6	NA	NA	NA

NA = Not applicable

^aConcentration (µg/ml) that produces a 50% inhibitory effect on radial mycelia growth.

^bMinimum inhibitory concentration (µg/ml).

Figure 1: Effect of essential on the mycelia growth of test fungi at different concentrations. (S. s = *S. sclerotiorum*, A. t = *A. tenuis*, C. g = *C. graminicola*, R. s = *R. solani* and F. o = *F. oxysporum*)

3.1.3. Spore germination assay

The results shown by the essential oil against spore germination are summarized in **table 5, Fig. 2**. The oil effected the germination of spores differently at different concentrations. The spore germination was not inhibited by Dimethyl sulphoxide (DMSO, 10% v/v) used as control. The spore germination inhibition ranges from 18.65% - 80.25% for the test pathogens. The spores of *F. oxysporum* were

found least susceptible to the essential oil of *B. stracheyi* and the spores of *C. graminicola* were most inhibited at the test range concentration. The IC₅₀ values for the spore germination of these pathogenic fungi were found 899.67µg/ml, 545.27µg/ml and 2014.7µg/ml for *A. tenuis*, *C. graminicola* and *F. oxysporum* respectively. The spore germination inhibition by the oil on comparison with the fungicide (standard) showed a significant effect (**table 6**).

Table 5: % spore germination inhibition by essential oil from *B. stracheyi*

Conc. $\mu\text{g/ml}$ → Pathogenic fungi ↓	250 $\mu\text{g/ml}$	500 $\mu\text{g/ml}$	1000 $\mu\text{g/ml}$	2000 $\mu\text{g/ml}$
<i>A. tenuis</i>	32.55	40.62	52.15	78.75
<i>C. graminicola</i>	36.24	52.30	68.15	80.25
<i>F. oxysporum</i>	18.65	26.56	31.75	49.95

Table 6: IC_{50} and MIC values of essential oil and fungicides (standard check) against spore germination of test pathogens

Pathogenic Fungi	Essential oil		Fungicide (standard check)			
	IC_{50}^a	MIC^b	Carbendazim		Mancozeb	
			IC_{50}^a	MIC^b	IC_{50}^a	MIC^b
<i>A. tenuis</i>	899.6	NA	68.3	NA	68.6	500
<i>C. graminicola</i>	545.2	NA	38.6	500	NA	NA
<i>F. oxysporum</i>	2014.7	NA	NA	NA	38.7	NA

NA = Not applicable

^aConcentration ($\mu\text{g/ml}$) that produces a 50% inhibitory effect on spore germination.^bMinimum inhibitory concentration ($\mu\text{g/ml}$).

3.2. DISCUSSIONS

The steam-distillation of fresh plant material (4kg rhizome of *B. stracheyi*) yielded 2.1g of colorless (transparent) oil (0.05% by weight). In all 33 compounds were identified which constitutes about 90.10% of the oil. The oil was found rich in oxygenated sesquiterpenoids (43.06%) and higher oxygenated hydrocarbons (29.83%). Sesquiterpenoids (0.46%) and monoterpenoids (0.24%) were almost absent in the oil. Among oxygenated sesquiterpenes Cadinene <14-hydroxy- δ -> (38.36%) and Khusinol (2.06%) were the main components. The higher hydrocarbons include n-Hexadecanoic acid (10.60%), *cis* 9-hexadecenoic acid

(4.46%), Linoleic acid (2.85%), Octacosane (2.52%), Cetanol (2.26%), Oleic acid (2.14%), Nonacosane (1.86%), n-Pentacosane (1.66%) and Untriacontane (1.48%). The oxygenated monoterpenoids include α -Terpineol (1.10%) as main constituent. The oil also contains m-xylene (11.27%) as the major lower hydrocarbon and some trace compounds as other portion of the oil having less than 1% area. Natural products acts as a better source of bio-fungicides. Different solvent extracts from the rhizome of *Bergenia stracheyi* have shown antifungal activity¹⁸. The methanolic extract of *Bergenia stracheyi* showed weak activity (10%) against fungus *Fusarium solanum*¹⁹.

Figure 2: % spore germination inhibition by essential oil at different concentrations. (*A. t* = *A. tenuis*, *C. g* = *C. graminicola*, and *F. o* = *F. oxysporum*)

4. CONCLUSIONS

The essential oil from the rhizome has been found rich in oxygenated sesquiterpenoids and higher oxygenated higher hydrocarbons which may be responsible for its antifungal activity. However the synergic effect may also be responsible for its bioactivity. As a result of its antifungal activity it can be used as bio-fungicide which is more safe and eco-friendly as compared to synthetic fungicides.

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