ORIGINAL ARTICLE

Phytochemical Screening, *In vitro* Antifungal and Antioxidant Activity of Essential Oil from roots of *Rheum webbianum* Royle from Himalayan Region

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Abstract

Aim: The aim of our study is to evaluate the phytogenic chemical compounds and assess their antifungal and antioxidant activity of essential oil of *Rheum webbianum* Royle growing in greater Himalayan region. **Materials and Methods**: In the present study, the phytochemical constituents of essential oil were isolated by steam distillation and screened by gas chromatography (GC) and GC-mass spectrometry analysis in relation with their Kovats indices from *R. webbianum* is rich in oxygenated monoterpenoids and sesquiterpenoids. The essential oil was further evaluated for their antifungal activity by well diffusion method and antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl scavenging (DPPH) scavenging assay at various concentrations. **Results**: The major chemical constituent's were undecanone (19.72%), maaliol (13.03%), 4-vinyl guaiacol (10.40%), 2E-undecanal (7.81%), valerianol (6.87%), viridiflorol (4.7%), eucalyptol (2.87%), and eugenol (2.98%) as the major constituents. The oil from the aerial parts of *R. webbianum* has shown moderate antifungal and activity with 100% mycelia growth inhibition against *Alternaria alternata* at concentration of 2400 µg/ml and *Bipolaris maydis* and *Rhizoctonia solani* at concentration of 2800 µg/ml. However, *Fusarium oxysporum* was found less susceptible for this oil. The IC_{50} showed a range from 873.4 µg/ml to 1502.3 µg/ml as compared with standard fungicides with IC_{50} values ranging from 37.8 µg/ml to 88.6 µg/ml. The free radical scavenging activity of *R. webbianum* oil employed by *in vitro* assay methods like DPPH at concentration of 600 µg/ml was 83%, respectively. **Conclusion**: Our study showed that maaliol followed by *ρ*-vinylguaiacol as the major components in this oil which was absent in previous findings. The essential oil had potent antifungal and antioxidant activity, respectively.

Key words: Antifungal, antioxidant, essential oil, free radical, phytochemicals, *Rheum webbianum*

INTRODUCTION

The valley Jammu and Kashmir is regarded as hub for the medicinal plants. The people there were using these medicinal plants for their cure and preventing various diseases since ancient times. There are about total of 937 plant species belonging to 129 families have been reported from Jammu and Kashmir having traditional medicinal uses.[1] The genus *Rheum* belonging to family Polygonaceae represented by 48 genera and 1200 species with 12 genera in India distributed in Himalayan region between 2800 and 3800 m. The genus *Rheum* contains 50 species, of which 12 are present in India.[2] The *Rheum webbianum* is a perennial herbaceous plant which acquired the status of endangered category. It is used for fever, cough, and diarrhea, and menstrual and liver disorders.[3] It is also used to cure inflammatory diseases and oxidative stress related to injuries.[4] *Rheum emodi* has constituents such as anthraquinone and stilbene which confer anticancer, anti-inflammatory, antimicrobial, antiulcer, and hepatoprotective activities.[5] The methanolic and aqueous extract of *R. emodi* possessed antimicrobial

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Received: 04-03-2018
Revised: 04-04-2018
Accepted: 15-04-2018

activity against bacteria *Pseudomonas aeruginosa* and *Bacillus megaterium* and fungi *Fusarium solani* and *Aspergillus flavous* with zone of inhibition ranging between 0.9 and 1.8 cm.[6] Methanolic and aqueous extracts of *Rheum ribes* Linn. Leaves possess anti-ulcer activity using pylorus ligation method at concentration 200 mg/kg produces a protective effect on ulcer-induced models.[7] The root extracts of *R. emodi* exhibit antifungal activity against *Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans*, and *Trichophyton mentagrophytes* (MIC = 25250 µg/ml).[8] Root extract of *R. rhabarbum* has antibacterial, antiviral, hepatoprotective, anti-ulcer,[9] and aloe emodin of root extract has anti-inflammatory responses.[10] Rhein possesses antibacterial activity against *Escherichia coli* K12 with MIC value of 128 µg/ml.[11] Anthraquinones C-glycosides occur in the form of 10-hydroxy-cascaroside C, 10-hydroxy-cascaroside D, 10R-chrysaloin-1-O-*β*-D-glycoside, cascaroside C, cascaroside D, and cassialoin. Other compounds extracted was rutin, rheinal, rhein-11-O-*β*-D-glucopyranoside, torachrysone-8-*β*-D-glucoside, epicatechin, and auronols (corpusin and maesopsin).[12] The root extracts of *R. ribes* contain aloe emodin, chrysophanol, aloe emodin-8-*β*-O-glucoside, sennoside A, and rhaponticin has antimicrobial properties, whereas shoots of *R. ribes* contain quercetin, 5-deoxy-quercetin, quercetin-3-O-rhamnoside, quercetin-3-O-galactoside, auercetin-3-O-rutinoside, and quercetin-3-O-rutinoside.[13] Sulfemodin-8-O-*β*-D-glucoside, revandchinone-1, revandchinone-2, revandchinone-3, revandchinone-4, 6-methyl-rhein, and 6-methyl-aloe-emodin.[14] The chloroform and methanolic extracts from the roots and stems of *R. ribes* have antioxidant activity, in 1,1-diphenyl-2-picrylhydrazyl scavenging (DPPH) scavenging, superoxide anion radical scavenging, ferric reducing power, and cupric reducing power.[15] Our present work revealed with phytochemical investigation and their bioactivity of *R. webbianum* plant.

MATERIAL AND METHODS

Plant material

The fresh plant material was collected from high altitudes of Bhalessa (Doda), Jammu and Kashmir (India) at an elevated altitude of 3000 m in the month of August 2016. The roots were washed with cold water and their dead skin was skimmed off and was used for the extraction of oil. The preliminary plant identification was done by Prof. P. C. Pandey, Department of Botany, Kumaun University, Nainital. The plant was further confirmed by Botanical Survey of India, Dehradun, Voucher specimen *R. webbianum* Royle, Acc. No.118091 where herbarium of plant specimens has been deposited.

Isolation of essential oil

The essential oil was obtained by steam distillation of fresh plant material (8 kg roots) using copper still fitted with spiral glass condenser. The distillate was saturated with NaCl and extracted with hexane. The hexane extract is dried with sodium sulfate (Na_2SO_4) and the solvent removed with Rotovap at moderate pressure and 38°C temperature and stored at 4°C for further analysis. All chemical and reagents of analytical grade and were obtained from Merk, Mumbai, India.

Chemical and reagents

All chemicals and reagents used were of analytical grade. Hexane, anhydrous Na_2SO_4, dimethyl sulfoxide (DMSO), ether, ethanol, and sodium hypochlorite were obtained from Merk, Mumbai, India, whereas potato dextrose agar (PDA), potato dextrose broth (PDB), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, α-tocopherol, and dextrose (D-glucose) were obtained from HiMedia Pvt., Ltd., Mumbai India

Analysis of essential oil by gas chromatography (GC) and GC-mass spectrometry (MS) analysis

The analysis of the oil was done using a gas chromatograph (Shimadzu GC QP-2010) equipped with RTx-5 MS capillary column, 1009701 (30.0 m × 0.25 mm, film thickness: 0.25 µm). The oven temperature (50°C–280°C) was programmed at 50°C for first 2 min, 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. N_2 was used as the carrier gas at 113.0 mL/min. The injector temperature was 250°C, detector temperature 260°C, and the injection volume 0.5 µL 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 (30.0 m × 0.25 mm, film thickness: 0.25 µm). The oven temperature (50°C–280°C) was programmed at 50°C for first 2 min, 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. N_2 was used as the carrier gas. The injection volume 0.5 µL and split ratio were 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.

Identification of compounds

Identification of different chemical constituents of the essential oil was done by comparing their Retention indices/ Kovats indices in relation to a series of n-alkanes (C_6-C_{33}) indices on the RTx-5 MS capillary column, either with those of published data[16] or with authentic samples which were further supported by NIST and WILEY mass spectral library searches. The results are presented in Table 1.

Plant pathogenic fungi and their culture

The foliage born fungi were obtained Division of Plant of Pathology, FOA, Chatha, Sher-e-Kashmir University of

Table 1: Phytochemical composition of roots essential oil from *R. webbianum*

Compound	% in the oil	Method of identification	
		R.I.[a]	Others
p-cymene	0.65	1025	b, c, d
limonene	1.52	1029	b, c, d
1,8-cineole	1.84	1032	c, d
Z-*β*-ocimene	0.48	1039	b, c, d
β-epoxystyrene	0.50	1045	c, d
undecanone	19.72	1090	b, c, d,
linalool	0.44	1098	b, c, d
n-nonenal	0.21	1102	c, d
cis-thujone	0.20	1106	c, d
terpinen-4-ol	1.57	1178	b, c, d
α-terpineol	1.23	1190	c, d
linalool acetate	0.35	1256	c, d
2-decanal	0.48	1266	c, d
4-ethylguaiacol	0.42	1277	c, d
bornyl acetate	1.76	1288	b, c, d
ρ-vinyl-guaiacol	10.40	1310	c, d
eugenol	2.98	1360	b, c, d
2*E*-undecenal	7.81	1364	b, c, d
α-copaene	0.57	1378	c, d
E-caryophyllene	1.25	1423	b, c, d
trans-*β*-bergamotene	0.48	1436	c, d
9-epi-E-caryophyllene	0.29	1468	b, c, d
β-patchoulene	3.26	1472	c, d
α-bulnesene	0.48	1493	c, d
β-selinene	0.54	1498	c, d
kessane	0.34	1532	c, d
maaliol	13.03	1540	c, d
caryophyllene oxide	0.13	1585	b, c, d
Diethyl phthalate	2.92	1591	c, d
viridiflorol	4.21	1596	b, c, d
β-eudesmol	2.73	1652	b, c, d
valerianol	6.87	1661	c, d
longifolol	0.26	1716	b, c, d
neocinidilide	0.30	1722	c, d
Z-nuciferol	0.47	1730	c, d
isofenchol	0.35	1804	c, d
phytone	0.48	1842	c, d
isobutyl phthalate	2.22	1906	c, d
elaol	2.16	1989	c, d
eicosane	1.66	2004	c, d
n-henicosane	3.80	2103	c, d
n-tetracosane	0.60	2185	c, d
Sesquiterpenoids	9.56		
Oxygenated sesquiterpenoids	59.04		
Monoterpenoids	3.67		
Oxygenated monoterpenoids	22.27		
Total identified	94.54		
Yield	6.6 g (4.05% by weight)		

[a]Retention index (RI) relative to homologous series of n-alkane (C_6 - C_{32}) on RTx-5MS Capillary Column. [b]Compound checked by authentic standards compounds. [c]Retention index (RI). [d]MS, NIST08.LIB, and WILEY8.LIB libraries spectra and the literature. *R. webbianum: Rheum webbianum*

Agricultural Sciences & Technology, Jammu (SKUAST-J). The pure culture of fungal and species was maintained in PDA and stored below 4°C. The pathogenic fungi, namely *Bipolaris maydis, Rhizoctoina solani, Alternaria alternata*, and *Fusarium oxysporum*, were cultured on PDA medium in sterilized Petri dishes.

In vitro antifungal assay

Poisoned food technique[17] using PDA as a medium was used to check the antifungal growth of oil against test fungi. The different concentration of essential oil was prepared by dissolving appropriate amount of oil in 10% DMSO and double-distilled water into 20 ml of PDA to obtain the desired concentration.[18] Mycelial plugs from edges of each culture were placed in the center of each PDA plate. The prepared plates were inoculated aseptically with assay discs test fungus was inoculated at 25 ± 2°C for 3–8 days until growth reached at periphery of the plate. Growth inhibition of each fungal strain was calculated as percentage inhibition of radial growth relative to control using the formula as

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

Where C is the concentration of control plate and T is the radial growth of test plate. The plates were used in triplicate for each treatment. IC_{50} values were graphically obtained from dosage response curves based on measurement at different concentrations.

Determination of minimum inhibitory concentration (MIC)

The MIC of oil was determined by agar diffusion method.[19] The oil was dissolved on 10% DMSO. A 10 µl of

spore suspension of each fungal strain was inoculated in a test tube in PDB medium and inoculated for 3–8 days. The control tubes containing PDB medium were inoculated only with fungal suspension. The MIC is the minimum concentration of oil in µg/ml where no visible growth is observed.

In vitro antioxidant activity

The DPPH assay was done according to the method described by Brand-Williams *et al.* 1995.[20] The DPPH 0.0039 g was dissolved in ethanol and made up to 100 ml with double-distilled water. The ethanol (20%) 20 ml and 80 ml double-distilled water was prepared. The 100 µM DPPH (50 µl) was added to equal volume of 20% ethanol to generate 400 µl DPPH. The oil samples of different concentrations were taken in different test tubes and added DPPH 400 µl and make volume up to 100 µl with double-distilled water. Then, it was shaken vigorously and taken in the dark for 20 min at room temperature. The reduction in absorbance was recorded at 520 nm in ultraviolet (UV)-visible spectrometer. Ascorbic acid and α-tocopherol was used as standard and controlled absorbance of DPPH was taken without adding oil sample and all the assays was carried out in triplicate. Scavenging effect (%) of free radical DPPH was calculated as:

$$\text{Scavenging effect \%age} = \frac{\text{Absorbance of control-Absorbance of oil sample}}{\text{Absorbance of control}} \times 100$$

IC_{50} assay was calculated graphically using curve by plotting antioxidant capacity or percentage inhibition versus corresponding sample concentrations.

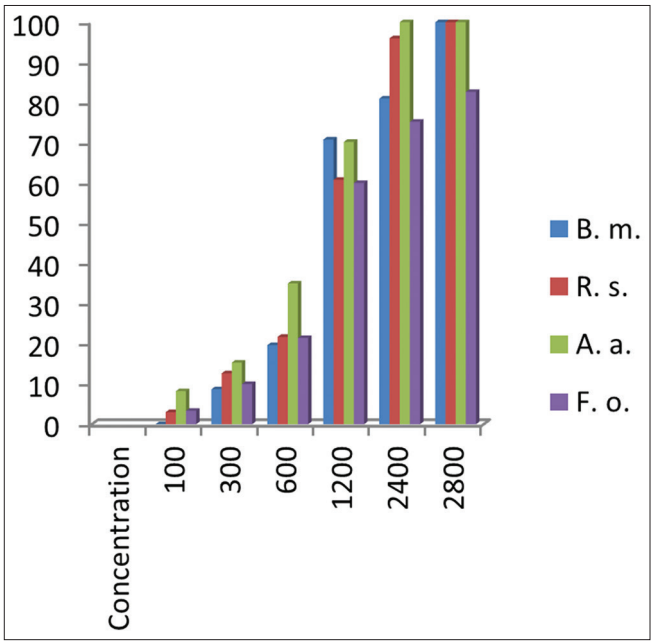

Figure 1: Effect of essential oil on mycelia growth of test fungi at different concentrations. (B. m. - *Bipolaris maydis*, R. s. - *Rhizoctonia solani*, A. a. - *Alternaria alternate*, and F. o. - *Fusarium oxysporum*

RESULTS AND DISCUSSION

The steam distillation of fresh plant material (8 kg) gives 6.5 g of oil yield with dark yellow in color and unpleasant smell. This oil was dominated by oxygenated sesquiterpenoids and oxygenated monoterpenoids whereas monoterpenoids and sesquiterpenoids constituted as minor components. Among oxygenated sesquiterpenoids maaliol (13.03%), *ρ*-vinylguaiacol (10.40%), 2*E*-undecanal (7.81%), valerianol (6.87%), viridiflorol (4.7%), *Z*-nuciferol (4.21%), and *β*-eudesmol (2.73%) were the major compounds whereas

Table 2: % mycelia growth inhibition[a] by essential oil from roots of *R. webbianum*

Pathogenic fungi (µg/ml)	100	300	600	1200	2400	2800
B. maydis	00.00±0.00	08.71±0.05	19.70±0.50	70.80±0.08	81.00±0.70	100.00±0.00
R. solani	03.05±0.01	12.65±0.05	21.73±0.30	60.79±0.10	96.01±0.06	100.00±0.00
A. alternata	08.22±0.20	15.33±0.03	35.06±0.03	70.21±0.90	100.00±0.00	100.00±0.00
F. oxysporum	03.34±0.60	10.00±0.20	21.44±0.30	60.02±0.06	75.22±0.90	82.65±0.80

[a]values within column are given as mean SD of three experiments. *R. webbianum: Rheum webbianum. B. maydis: Bipolaris maydis, R. solani: Rhizoctonia solani, A. alternate: Alternaria alternata, F. oxysporum: Fusarium oxysporum*

Table 3: IC50 and MIC values of essential oil and fungicides (positive control) against test pathogens

Pathogenic fungi	Essential oil		Fungicide (positive control)			
			Amphotericin		Clotrimazole	
	IC_{50}[a]	MIC[b]	IC_{50}[a]	MIC[b]	IC_{50}[a]	MIC[b]
B. maydis	940.6	2800	NA	600	NA	1200
R. solani	1502.3	2800	37.8	NA	42.2	NA
A. alternata	873.4	2400	NA	NA	88.6	1200
F. oxysporum	1260.2	NA	65.3	600	NA	600

NA: Not appeared. [a]Concentration that produces 50% inhibitory effect on mycelia growth. [b]Minimum inhibitory concentration (g/ml).
B. maydis: Bipolaris maydis, R. solani: Rhizoctonia solani, A. alternate: Alternaria alternata, F. oxysporum: Fusarium oxysporum

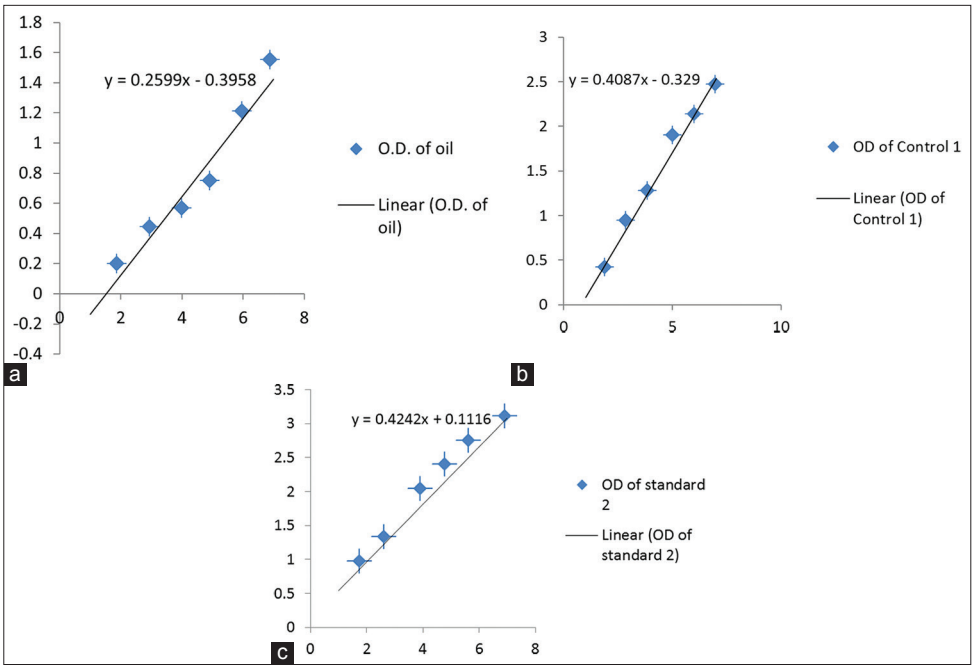

Figure 2: (a-c) Effect of different concentrations of essential oil and controls on 1, 1-diphenyl-2-picrylhydrazyl scavenging assay

undecanone (19.72%), 1,8-cineole (1.83%), terpinen-4-ol (1.57%), and α-terpineol (1.23%) were major among oxygenated monoterpenoids [Table 1]. The maaliol and ρ-vinylguaiacol were the major constituents which were absent in previous findings. This change in composition is due to environmental adaptation, climatic conditions, and altitude factor of the plants.

The oil inhibits the growth of mycelia strains in a dose-dependent manner. The essential oil show varied effect at different concentrations. The oil was found to be effective for all the pathogenic test fungi. The inhibitory effect of oil varies from 8.71% to 100.00%. The oil of *R. webbianum* completely inhibits the mycelia growth of *A. alternata* at 2400 µg/ml and *B. maydis* and *R. solani* at 2800µg/ml whereas *F. oxysporum* is to be less susceptible for this oil [Table 2 and Figure 2]. The essential oil of this plant is endowed with antibacterial, antiviral, antiulcer, and anti-inflammatory agent. The natural product acts as potent biofungicides and antibacterial agents. The reports on antimicrobial activity of *R. emodi* against *Bacillus subtilis* and *P. aeruginosa* showed potent ZOI ranges from 0.7 to 1.4 cm,[8] and rhizome extract has antifungal activity against *Aspergillus niger* and *C. albicans*.[21] The investigations on antimicrobial activity of different plant extract against plant pathogens have been performed worldwide,[22,23] hence our result have important because it provides information about the essential oil. Our work dealed with antifungal activity of essential oil from roots of *R. webbianum*. The oil had shown potent effect against *A. alternata, R. solani,* and *Bipolaris maydis* at concentrations of 2400 µg/ml and 2800 µg/ml, respectively [Table 2 and Figure 1]. The result of our studied research work showed that oil is safe potent and effective biofungicide.

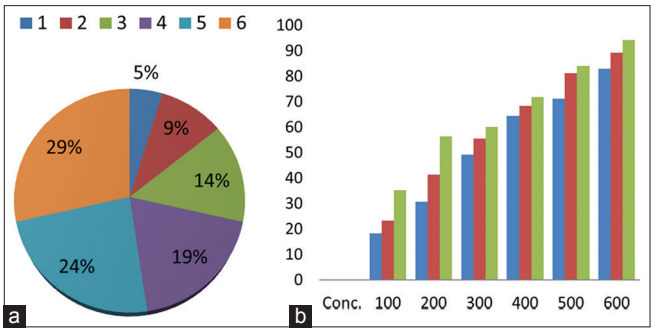

Figure 3: (a and b) %age inhibition of 1, 1-diphenyl-2-picrylhydrazyl scavenging assay shown by Pie-chart and Histogram at various concentrations. (%age inhibition= essential oil, control 1= ascorbic acid, control 2= α-tocopherol)

Table 4: Absorbance shown by the essential oil and control (Ascorbic acid and Vitamin E) at different concentrations

Concentration (µg/ml)	Absorbance		
	Essential oil	Ascorbic acid	α-tocopherol
100 µg/ml	0.201±0.010	0.236±0.022	0.921±0.021
200 µg/ml	0.437±0.002	0.280±0.032	1.320±0.004
300 µg/ml	0.563±0.030	0.520±0.002	1.890±0.002
400 µg/ml	0.736±0.006	0.826±0.001	2.348±0.006
500 µg/ml	1.193±0.012	1.890±0.043	2.613±0.013
600 µg/ml	1.536±0.001	2.520±0.001	3.124±0.042

Values in the column are men of three absorbance results±SD

This study affirms that *in vitro* antioxidant activity of essential oil from roots of *R. webbianum* was comparable to

Table 5: Percentage scavenging activity of different concentration of essential oil and control

Conc. (µg/ml)	%age of DPPH scavenged by Essential oil	%age of DPPH scavenged by Ascorbic acid	%age of DPPH scavenged by α-Tocopherol
100	18.3	23.4	35.2
200	30.8	41.2	56.4
300	49.3	55.6	60.2
400	64.5	68.3	71.8
500	71.2	71.2	84.00
600	83.00	89.3	94.3

DPPH: 1, 1-diphenyl-2-picrylhydrazyl scavenging

those of standard ascorbic acid and Vitamin E [Table 5].[15,24] Higher absorbance indicated higher reducing power. The present study indicated that the oil at concentration of 600 µg/ml could play an important role in the management of oxidative stress. Thus, it was considered that the essential oil had antioxidant activity against DPPH radical [Tables 3, 4 and Figure 3].

Statistical analysis

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

CONCLUSIONS

This study showed that malliol followed by *ρ*-vinyl-guaiacol as the major components in this oil which was absent in previous findings. The essential oil from the roots has been responsible for its antifungal activity. However, the synergic effect is found to be responsible for its bioactivity. As a result of its antifungal activity, it can be used as biofungicides and which is more safe and eco-friendly as compared with synthetic chemical fungicides. This oil has therapeutic potential as natural antioxidant in reducing free radical scavenging action.

ACKNOWLEDGMENTS

We are highly thankful to Prof. P. C. Pandey, Kumaun University, Nainital and Kumar Ambrish, Botanical Survey of India, Dehradun, India, for plant identification. We are also thankful to Prof. A. B. Melkani, HOD, Chemistry, and Prof. S. P. S. Mehta, D. S. B. Campus, KU, Nainital, and Dr. Sachin Gupta, Sher-e-Kashmir University of Agricultural sciences and Technology, Jammu for providing necessary facilities. Furthermore, we are thankful to Dr. Rishendra Kumar, Department of Biotechnology, Bhimtal Campus, Nainital (India), for their valuable necessarily guidance.

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Source of Support: Nil. **Conflict of Interest:** None declared.