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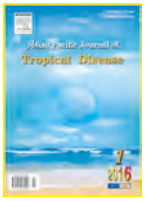

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In vitro antimicrobial efficacy of *Rhynchostegium vagans* A. Jaeger (moss) against commonly occurring pathogenic microbes of Indian sub-tropics

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ABSTRACT

Objective: To study the antimicrobial effect of organic extracts with a standard dose of *Rhynchostegium vagans* (*R. vagans*) on pathogenic bacteria and fungi.
Methods: *R. vagans* was extracted in solvents (ethanol and acetone) and the extracts were evaluated for antimicrobial activity by using disc diffusion assay. Minimum inhibitory concentration and minimum bactericidal/fungicidal concentration was observed by employing micro broth dilution method. Mode of inhibition of ethanolic extract against *Aspergillus flavus* var. *columnaris* (*A. flavus* var. *columnaris*) was assessed by scanning electron microscopy.
Results: It was found that the ethanolic extract of *R. vagans* was the most potent with lowest minimum inhibitory concentration (3.91 to 61.25 μg/mL) and minimum bactericidal/fungicidal concentration (3.91 to 500 μg/mL), respectively. Significant morphological and ultrastructural alterations were seen in *A. flavus* var. *columnaris*. Among microorganisms, Gram negative bacteria (*Escherichia coli*, *Erwinia chrysanthemi* and *Salmonella enterica*) and fungi (*A. flavus* var. *columnaris* and *Aspergillus parasiticus* var. *globosus*) were found more sensitive. Ethanolic extract was found superior over the antibiotics (chloramphenicol and fluconazole).
Conclusions: *R. vagans* exhibited effective antimicrobial activity against all the microorganisms. The moss can be used as a broad spectrum herbal antimicrobial agent in pharmaceutics.

1. Introduction

Plant drugs or botanicals are one of the principle sources of pharmaceutical agents used in orthodox medicine[1]. Most of the plant drugs have been derived from higher plants; very few of them are from non vascular plants like bryophytes. Bryophytes are the group of the oldest known land plants. They are ubiquitously present in variety of habitats ranging from desert to tropical rainforests, from sea shore to alpine, and from soil to water, where chances of occurrence of different microbes are prominent. They lack any protective shield around their body. A single wall epidermis and parenchymatous cortex is not enough to cope with the different environmental conditions. Therefore, they have devised an effective strategy of developing remarkable chemical weapons against infectious and stressful environment. These chemical weapons are the secondary metabolites–sesquiterpenoids, phenols, flavonoids, isoflavonoids and bis(bibenzyls), *etc*.

Bryophytes are known to be used in ethnobotany to cure diseases of both humans and animals[2]. They have also been used as medicinal plants to cure cuts, burns, external wounds, bacteriosis, pulmonary tuberculosis, fractures, convulsions, scalds, uropathy and pneumonia, *etc*.[3]. They also serve as a significant and promising source of antibiotics and bioactive compounds in nature[4]. In recent years, many bryophytes *viz*., *Targionia hypophylla*, *Plagiochasma appendiculatum*, *Rhodobryum giganteum*, *Marchantia polymorpha* and *Dumortiera hirsuta* and many more have been explored for their antibiotic activity[2,3,5-7].

Rhynchostegium vagans (*R. vagans*), a light green moss of the family Brachytheciaceae, is well flourishing moss in the Kumaon Himalayas occurring together with *Plagiothecium denticulatum* and

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Drepanocladus exanulatus[8]. It has wide distribution from foothills to mid hills of Himalayas occurring near watery habitats. Nowhere the plant is seen affected by any disease, though growing near aquatic habitats. This tempted us to study the antimicrobial effect of organic extracts of a standard dose of *R. vagans* on pathogenic bacteria and fungi.

2. Materials and methods

2.1. Collection of plant materials

Plants of *R. vagans* (Brachytheciaceae) were collected during the months of June to July 2012 from Chaubatia (Ranikhet) in Kumaon Himalayas of Uttarakhand (1 800 m, 29°38'612" N and 79°25'245" E). The plant was identified by Dr. SD Tewari. A voucher specimen of the plant was submitted to the herbarium of Department of Biological Sciences.

2.2. Preparation of plant organic extracts

The plants were thoroughly washed under running tap water, shade dried, pulverized and extracted by cold percolation method (10 g/100 mL) in 80% ethanol and acetone. The extract was filtered and concentrated by using rotary evaporator (Biogen). Different concentrations of crude extract (100, 400, 700 and 1 000 μg/mL) were prepared and used for further study.

2.3. Microbial strains

Different microoganisms *viz*., *Pseudomonas aeruginosa* (MTCC 424) (*P. aeruginosa*), *Staphylococcus aureus* (MTCC 902) (*S. aureus*), *Bacillus cereus* (MTCC 430) (*B. cereus*), *Aspergillus flavus* var. *columnaris* (MTCC 1973) (*A. flavus* var. *columnaris*) and *Aspergillus parasiticus* var. *globosus* (MTCC 411) (*A. parasiticus* var. *globosus*) were procured from Institute of Microbial Technology, Chandigarh. *Escherichia coli* (*E. coli*) and *Salmonella enterica* (*S. enterica*) were kindly provided by Public Health Department, College of Veterinary Sciences, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar. The plant pathogens *Erwinia chrysanthemi* (*E. chrysanthemi*) and fungi [*Fusarium oxysporum* f. sp. *lycopersci* (*F. oxysporum* f. sp. *lycopersci*), *Colletotrichum falcatum* (*C. falcatum*) and *Rhizoctonia solani* (*R. solani*)] were provided by Department of Plant Pathology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar.

2.4. Antimicrobial assay

Disc diffusion assay was used for evaluation of antimicrobial activity[6]. In assay for antibacterial activity, the nutrient agar plates of bacteria treated with organic extracts (40 μL into each disc) of different concentrations were incubated at (37 ± 2) °C for 24 h. Antibacterial activity of the plant extracts was determined by measuring the zone of inhibition (ZI) in mm against all bacteria. The antibiotics as positive controls (streptomycin, tetracycline and choloramphenicol) were used for comparison with the extracts regarding antibacterial activity; fungicides (carbendazim for *F. oxysporum* f. sp. *lycopersci* and and *C. falcatum* and fluconazole for *A. flavus* var. *columnaris* and *A. parasiticus* var. *globosus*) were used as positive control for antifungal activity, and respective solvents as negative control.

All the fungi were screened through disc diffusion except *A. flavus* var. *columnaris* and *A. parasiticus* var. *globosus*, because of lacking uniformity in their growth pattern. In assay for antifungal activity, potato dextrose agar was poured aseptically in the plates and kept for solidification at (28 ± 2) °C for 72 h. Four discs, two treated with plant extracts and two controls along with the test fungus were kept in same Petri plate. % Inhibition of fungal growth was calculated by the following formula:

% Inhibition = Mycelial growth (control) - Mycelial growth (treatment)/Mycelial growth (control) × 100

where, mycelial growth was determined by measuring the diameter of the fungus both in control and treatment.

2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) of organic extracts

Micro broth dilution assay was done to determine both inhibitory and bactericidal/fungicidal concentration of organic extracts[9]. Freshly prepared nutrient broth for bacteria and potato dextrose broth for fungi were used as diluent. Fresh and revived culture of test microorganisms were diluted 100 folds in broth (100 μL of microorganism in 10 mL broth). For inoculation of culture, CFU was determined and was found to be 1×10^6 CFU/mL for bacteria, while it was 1×10^9 CFU/mL for fungi by taking optical density at 620 nm using UV-visible spectrophotometer (Genesys). Decreasing concentrations of the plant extract (1 000 to 0.98 μg/mL) in two fold dilution series were added to the test tubes containing the fresh microorganism cultures.

All tubes with bacterial and fungal organisms were incubated at 37 °C for 24 h and 28 °C for 72 h, respectively. Visible turbidity and optical density of cultures were determined at 620 nm by using UV-visible spectrophotometer. The lowest concentration that inhibited visible growth of tested organisms was recorded as MIC, and that caused no visible microbial growth was considered as MBC.

All the experiments were performed in triplicates. Values were expressed as mean ± SE. ANOVA revealed level of significance at $P < 0.05$ among different microorganisms and different extracts by using Dunkan's multiple range test.

2.6. Scanning electron microscopy (SEM) analysis

Effect of ethanolic extract of *R. vagans* on *A. flavus* var. *columnaris* was observed by SEM following the protocol with minor modifications[10]. Fresh *A. flavus* var. *columnaris* culture was incubated at 28 °C in potato dextrose broth (for 3 days) with ethanolic extract (at MIC and MFC). The mycelia treated with the

solvent (ethanol) were used as negative control. The sample of *A. flavus* var. *columnaris* was prepared by fixing in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer for 1 h at room temperature. After washing with the buffer (pH 7.2), the fungal sample was fixed in the same buffer for 0.5 h at room temperature. The specimens were dehydrated in a series of graded ethanol (50, 60, 70, 80, 90 and 100%) for a period of 5 min in each grade. For the purpose of drying, the dehydrated samples were kept under light for 3 days on a glass slide. Fixed samples were critical point dried under carbon dioxide and sputter-coated with gold. Morphological changes of the fungal cells were observed by using SEM (JEOL 6610LV) at Govind Ballabh Pant University of Agriculture and Technology, Pantnagar.

3. Results

The results obtained showed that most of the test microorganisms were sensitive to the organic extracts of *R. vagans* in dose-dependent manner. Ethanolic extract showed higher antimicrobial activity than acetonic extracts (Tables 1–3). A broader spectrum of inhibition was showed by *R. vagans* towards bacteria than towards fungi (Tables 1 and 2). At maximum concentration of 1 000 μg/mL, the ZI against different bacteria ranged from (14.33 ± 0.33) to (29.00 ± 0.57) mm. Among bacteria, the Gram negative bacteria were more sensitive than Gram positive bacteria to increasing concentrations of ethanolic extracts of the moss. The similar trend of inhibition was observed in acetonic extract with maximum ZI for *E. coli* [(22.00 ± 0.57)] mm and *E. chrysanthemi* [(21.33 ± 0.57) mm]. However, acetonic extract did not show any inhibition against *S. enterica* and *S. aureus*. Ethanolic extract showed highest ZI against *E. coli* [(29.00 ± 0.57) mm] followed by *E. chrysanthemi* [(22.67 ± 0.33) mm] and *S. enterica* [(21.00 ± 0.57) mm] (Table 1). In *E. coli*, the ZI of ethanolic extract of the moss was higher than the that of streptomycin and tetracycline. The ZI of ethanolic extract of the moss was higher than that of chloramphenicol against all the tested microorganisms except *S. aureus* and *P. aeruginsoa* (Table 1).

The ZI against the fungi by the treatment and control significantly differed from each other in the same plate of potato dextrose broth as shown in Figure 1 and Table 2. The ZI of *F. oxysporum* f. sp.

Table 1

Antibacterial activity of *R. vagans* extracts (ethanol and acetone) against different bacteria compared to standard drugs (40 μg/mL).

Treatments	Concentration (μg/mL)	ZI of different bacteria (mm)					
		S. aureus	*B. cereus*	*S. enterica*	*E. chrysanthemi*	*E. coli*	*P. aeruginosa*
Ethanol extract	100	11.67 ± 0.66^{e}	10.00 ± 0.57^{d}	14.00 ± 0.57^{e}	16.33 ± 0.33^{e}	19.67 ± 0.33^{d}	16.00 ± 0.57^{e}
	400	13.00 ± 0.57^{d}	15.33 ± 1.20^{b}	14.67 ± 0.33^{e}	17.67 ± 0.33^{d}	25.67 ± 0.33^{b}	16.67 ± 0.66^{ce}
	700	14.67 ± 0.33^{c}	15.33 ± 0.66^{b}	17.67 ± 0.33^{d}	19.33 ± 0.33^{c}	28.67 ± 0.66^{a}	18.00 ± 0.57^{cd}
	1 000	15.67 ± 0.33^{c}	16.33 ± 0.66^{b}	21.00 ± 0.57^{c}	22.67 ± 0.33^{b}	29.00 ± 0.57^{a}	19.33 ± 0.33^{c}
Acetone extract	100	0.00 ± 0.00^{f}	10.00 ± 0.00^{d}	0.00 ± 0.00^{g}	14.00 ± 0.57^{f}	17.67 ± 0.66^{e}	13.67 ± 0.33^{f}
	400	0.00 ± 0.00^{f}	11.33 ± 0.33^{cd}	0.00 ± 0.00^{g}	16.67 ± 0.33^{e}	19.33 ± 0.88^{d}	14.67 ± 0.33^{f}
	700	0.00 ± 0.00^{f}	12.67 ± 0.33^{c}	0.00 ± 0.00^{g}	19.00 ± 0.33^{c}	20.33 ± 0.33^{d}	16.00 ± 0.57^{e}
	1 000	0.00 ± 0.00^{f}	14.33 ± 0.88^{bc}	0.00 ± 0.00^{g}	21.33 ± 0.57^{c}	22.00 ± 0.57^{c}	17.00 ± 0.55^{c}
Streptomycin	40	29.67 ± 0.33^{a}	25.33 ± 0.33^{a}	26.33 ± 0.33^{b}	29.00 ± 0.57^{a}	25.67 ± 0.33^{b}	26.00 ± 0.57^{b}
Tetracycline	40	28.67 ± 0.66^{a}	26.33 ± 0.33^{a}	31.67 ± 0.33^{a}	28.33 ± 0.33^{a}	28.67 ± 0.66^{a}	30.33 ± 0.33^{a}
Chloramphenicol	40	28.00 ± 0.33^{ab}	15.33 ± 0.33^{b}	12.00 ± 0.57^{f}	14.33 ± 0.33^{f}	14.67 ± 0.33^{f}	20.00 ± 0.57^{c}
SEm		0.37	0.60	0.36	0.41	0.55	0.51
Critical differences at 5%		1.10	1.76	1.06	1.21	1.61	1.50
Coefficient of variation		5.06	6.60	5.02	3.61	4.10	4.70

The data with different superscripted alphabets in each column show significant difference with each other, while data with the same letter show non significant diference with each other by One-way ANOVA. Level of significance was observed at $P < 0.05$. SEm: The standard error of the mean estimates the variability between samples.

Table 2

Antifungal activity of *R. vagans* extracts (ethanol and acetone) against different microorganisms compared to fungicide (carbendazim) at 100 μg/mL.

Treatments	Concentration (μg/mL)	*F. oxysporum* f. sp. *lycopersici*		*C. falcatum*		*R. solani*		*A. flavus* var. *columnaris*	*A. parasiticus* var. *globosus*
		ZI (mm)	% Inhibition	ZI (mm)	% Inhibition	ZI (mm)	% Inhibition	ZI (mm)	ZI (mm)
Ethanol	100	0.00 ± 0.00^{f}	0.00	90.00 ± 0.57^{ab}	3.26	0.00 ± 0.00	0.00	ND	ND
	400	22.00 ± 0.33^{c}	41.07	86.00 ± 0.57^{c}	8.60	0.00 ± 0.00	0.00	ND	ND
	700	19.67 ± 0.11^{d}	49.10	76.67 ± 1.20^{e}	13.18	0.00 ± 0.00	0.00	ND	ND
	1 000	19.00 ± 0.00^{d}	51.23	52.67 ± 0.88^{f}	33.75	0.00 ± 0.00	0.00	ND	ND
Acetone	100	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	ND	ND
	400	29.67 ± 0.29^{b}	34.73	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	ND	ND
	700	23.00 ± 0.33^{c}	41.02	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	ND	ND
	1000	20.67 ± 0.11^{cd}	47.02	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00	ND	ND
Carbendazim	100	17.00 ± 0.57^{e}	69.49	21.00 ± 0.57^{g}	77.52	ND	ND	ND	ND
Negative control		57.67 ± 0.88^{a}		90.00 ± 0.57^{ab}		ND	ND	ND	ND
SEm		0.45		0.79					
Critical differences at 5%		1.35		2.34					
Coefficient of variation		3.81		1.77					

The data with different superscripted alphabets in each column show significant difference with each other, while data with the same letter show non significant diference with each other by One-way ANOVA. ND: Not determined. SEm: The standard error of the mean estimates the variability between samples; Negative control: Respective solvents.

lycopersici and *C. falcatum* was decreased while % inhibition was increased with the increasing concentration of the ethanolic extract of *R. vagans*. The % inhibition of *F. oxysporum* f. sp. *lycopersici* ranged from 34.73% to 51.23% which was well comparable to the % inhibition (69.49%) of carbendazim (Table 2). Similarly, the acetonic extract showed dose dependent activity against *F. oxysporum* f. sp. *lycopersici* but was ineffective against *C. falcatum* (Table 2).

Table 3
MIC, MBC/MFC of *R. vagans* extracts (ethanol and acetone) against different microorganisms. µg/mL.

Microorganisms	Ethanol extract		Acetone extract		Fluconazole
	MIC	MBC/MFC	MIC	MBC/MFC	MIC/MFC
S. aureus[a]	62.50	125.00	-	-	ND
B. cereus[a]	62.50	125.00	125.00	250.00	ND
E. chrysanthemi[b]	7.81	7.81	15.62	31.25	ND
E. coli[b]	3.90	3.90	3.90	7.81	ND
P. aeruginosa[b]	31.25	62.50	62.50	250.00	ND
F. oxysporum f. sp. *lycopersici*	125.00	250.00	250.00		ND
C. falcatum	125.00	500.00	-	-	ND
A. flavus var. *columnaris*	15.62	62.50	62.50	125.00	31.25
A. parasiticus var. *globosus*	31.25	250.00	125.00	250.00	31.25
R. solani	-	-	-	-	ND

-: No inhibition; ND: Not determined; [a]: Gram positive; [b]: Gram negative.

The MIC and MBC/MFC ranged from 3.90 to 250.00 µg/mL and 3.90 to 500.00 µg/mL, respectively, against different microorganisms (Table 3). The MIC and MBC of extract were lower than MIC, MFC of extract. For bacteria, the lowest MIC/MBC (3.90/3.90 µg/mL) were found in ethanolic extract against *E. coli* followed by *E. chrysanthemi* (MIC/MBC = 7.81/7.81 µg/mL). Among fungi, *A. flavus* var. *columnaris* was found most sensitive with lowest MIC/MFC (15.62/62.50 µg/mL) followed by *A. parasiticus* var. *globosus* with lowest MIC/MFC of 31.25/250.00 µg/mL to ethanolic extract of the plant. Fluconazole showed similar MIC and MFC of 31.25 µg/mL against *A. flavus* var. *columnaris* and *A. parasiticus* var. *globosus*.

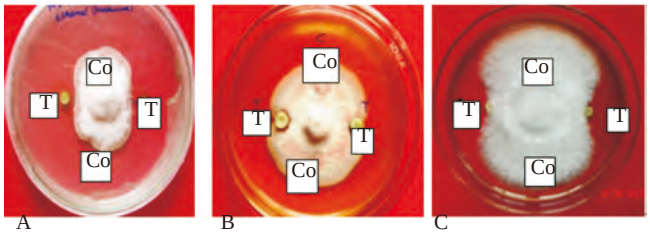

Figure 1. Antifungal activity of *R. vagans*.
A: Disc diffusion assay of ethanolic extract against *F. oxysporum* f. sp. *lycopersici*; B: Disc diffusion assay of acetonic extract against *F. oxysporum* f. sp. *lycopersici*; C: Disc diffusion assay of ethanolic extract against *C. falcatum*; Co: Control; T: Treatment.

The antifungal activity of the ethanolic extract was also confirmed by using SEM imaging of *A. flavus* var. *columnaris* (Figure 2). The control sample of *A. flavus* var. *columnaris* showed normal and intact tubular structure of hyphae with smooth cell wall (Figure 2A). At MIC (15.62 µg/mL) hyphae were slightly wrinkled and distorted (Figure 2B). The MFC (62.50 µg/mL) treated conidium (present in conidiophore) of *A. flavus* var. *columnaris* was trigonal, perforated and distorted while the control conidium was oval, smooth without any distortion and perforation (Figures 2C and 2D). The perforation in the conidia was found due to absence of cytoplasm as shown in Figure 2D.

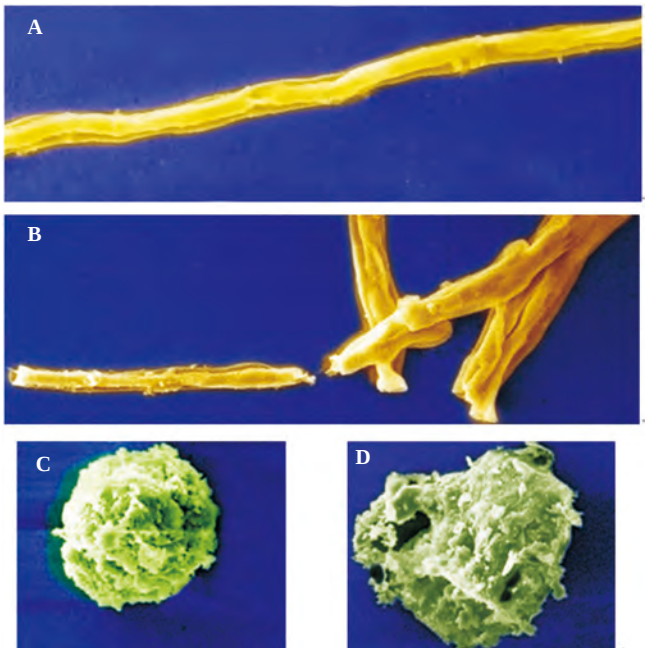

Figure 2. SEM imaging of *A. flavus* var. *columnaris*.
A: Normal hypha with smooth, thick and normal cell surface; B: Hypha treated with *R. vagans* (ethanolic extract) at MIC (15.62 µg/mL) with destructed cytoplasm and distorted cell wall; C: Conidium with completely intact, spherical and smooth surface; D: Conidium showing slightly trigonal, distorted and perforated structure with small pits when treated with MFC (62.50 µg/mL).

4. Discussion

In the present study, all the organic extracts of *R. vagans* showed strong and broad spectrum inhibition against *E. coli*, *E. chrysanthemi*, *B. cereus*, *P. aeruginosa*, *A. flavus* var. *columnaris*, *A. parasiticus* var. *globosus* and *F. oxysporum* f. sp. *lycopersici*. Earlier reports also suggested good antimicrobial activity in organic extracts of *Rhynchostegium riparioides* and *R. vagans*[11,12]. *E. chrysanthemi*, *S. enterica* and *E. coli* were found to be most sensitive bacteria while *A. flavus* var. *columnaris* and *A. parasiticus* var. *globosus* were most sensitive fungi. This broad spectrum antimicrobial activity of the moss extract is because of the presence of flavonoids, and other antimicrobial substances[13]. Lower values of bacterial MIC/MBC than the fungal MIC/MFC indicate that extracts are more effective against bacteria even at very low dosage. Similar values of MIC and MBC/MFC showed the presence of specific group of antibiotic compounds in the particular extract[14].

In consistent with the present study, several other studies have also shown good antifungal activity of organic extracts of bryophytes against *Aspergillus* sp.[15-18]. Microscopic examination revealed distorted structure of hyphae and perforated and deformed conidia. The results are supported by several other microscopic studies[17-19]. The distortion in the hyphal structure of *A. flavus* var. *columnaris* is consistent with the other studies in which hyphal cell wall of the *A. niger* revealed

significant alterations in the morphology when treated with *Citrus sinensis* oil and citronella oil, respectively[20,21]. The distortion of the hyphal structure can be due to change in hyphal cell permeability caused by the active component present in the extract resulting in leakage of cytoplasm[22]. Another study reported penetration of plasma membrane of *A. flavus* var. *columnaris* due to lipophilic character of the essential oil of *Ageratum conyzoides*[23]. Bryophytes also possess good antimicrobial activity due to presence of high level of phenols, flavonoids and mono-, sesqui-, and diterpenoids [18,24] which serve as lipophilic compounds[25]. These compounds like phenols or ploypheno ls are soluble in aqueous-ethanolic and methnaolic solvents and showed strong antimicrobial activity in plants[26,27]. In the present study, ethanolic extract showed higher antimicrobial activity than the acetonic extract did. The ethanolic extract was also found superior over the antibiotics chloramphenicol and fluconazole, and was equivalent to tetracycline, streptomycin and carbendazim. Results showed that antibiotic activity is dependent on the solvent system used for extraction as reported in other studies[7]. It is possible that the ethanolic extracts of *R. vagans* possess high amount of bioactive lipophilic compounds like, polyphenols, flavonoids and terpenoids which are able to be transported through the cell membrane and cause interference of the cellular metabolism of the microoganisms.

Effective control of large number of microbes by using organic extract of *R. vagans* suggests its immense potential in formulation of herbal drugs against both bacteria and fungi, providing good substitute to conventional antibiotics.

Conflict of interest statement

We declare that we have no conflict of interest.

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