



Phytochemistry and Antimicrobial activities of *Pogonatum microstomum* (R. Br. ex Schwägr.) Brid. (Bryophyta; Musci: Polytrichaceae)

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Abstract

Pogonatum microstomum belongs to family Polytrichaceae (Bryophyta; Musci). The phytochemical group test of three solvent extracts of this plant materials for the detection of secondary metabolites were performed by the standard methods. Flavonoids, glycosides, triterpenoids, phenols and sterols are present in all three extracts. Saponins present only in chloroform extract. Antimicrobial properties of various extracts was detected against two Gram – positive and seven Gram – negative human pathogenic bacteria and four dermatophytic fungi and it was determined by standard agar well diffusion method. The chloroform extract showed the higher degree of inhibition zone against, *Streptomyces pneumonia*, *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Agrobacterium tumefaciens*. Pet ether and chloroform extracts showed the higher degree of inhibition zone against *Candida albicans* and *Trichophyton rubrum*.

Keywords: polytrichaceae, phytochemistry, antimicrobial, secondary metabolites, bryophyte

Introduction

Even though bryophytes are the second largest taxonomic group in the plant kingdom, but studies conducted to better understand their chemical composition which are limited and scattered. The bryophytes are delicate land plants and are lacking of thick cuticle and bark therefore they have biochemically active compounds which perform defense mechanism to protect them from enemies like fungi, bacteria and insects.

Bryophytes contain numerous potentially useful compounds, including oligosaccharides, polysaccharides, sugar alcohols, amino acids, fatty acids, aliphatic compounds, phenylquinones and aromatic and phenolic substances, but much work remains to link medical effects with specific bryophyte species or compounds [20]. A number of unsaturated lipids, esters, flavonoids, tripenoids and phenols have been reported from bryophytes [2].

Bryophytes have been explored extensively for numerous active constituents and pharmacological activity. Antibacterial and anti-inflammatory activity [13], Cytotoxic, anticancer and antitumour activity [23, 24] in liverworts and mosses have been described. An evaluation of literature [19,5,4,11,2,3,8,7,17,18,14,6] indicates that different solvent extracts of about 150 species of hepatics and mosses were used different compounds isolation. They showed antimicrobial properties against several groups of fungi and yeasts, as well as against gram positive and negative bacteria.

Pogonatum microstomum (R. Br. ex Schwägr.) Brid. belongs to family Polytrichaceae and it contains one of the most medicinally important genera *Polytrichum*. One among well-

known species *Polytrichum commune* reduces inflammation and act as antipyretic, laxative and hemolytic agent [12, 9]. The oil extract from the hair cap moss *Polytrichum commune* is used to beautify and strengthen the hair [20].

Materials and Methods

Collection, Drying, Powdering and Preparation of extract of *Pogonatum microstomum*

Pogonatum microstomum was collected from Kigga regions (13° 24.57' N, 75° 10.54'E, and 721 m MSL) of Chikkamagaluru district comes under central part of the Western Ghats, India. The moss was identified and voucher specimens (BMCK323) have been deposited in Department of PG Studies and Research in Applied Botany, Kuvempu University, Shankaraghatta, Shivamogga, Karnataka, India. Prior to extraction, the plant material was separated from adhering soil particles, washed in running tap water and dried [5]. The dried samples were grinded to obtain fine powder.

Different solvents such as Petroleum ether, Chloroform and Methanol were selected for extraction of selected mosses based on their polarity or dielectric constant values and density.

The powdered material of 1kg each were packed in a soxhlet apparatus and with a range of solvents, starting with low polar solvents to high (petroleum ether, chloroform and methanol) 48h. The extracts were filtered by Whatman filter paper No.1, the filtrates were kept in aseptic condition for complete evaporation of solvent. The dried extracts were preserved in desiccator.

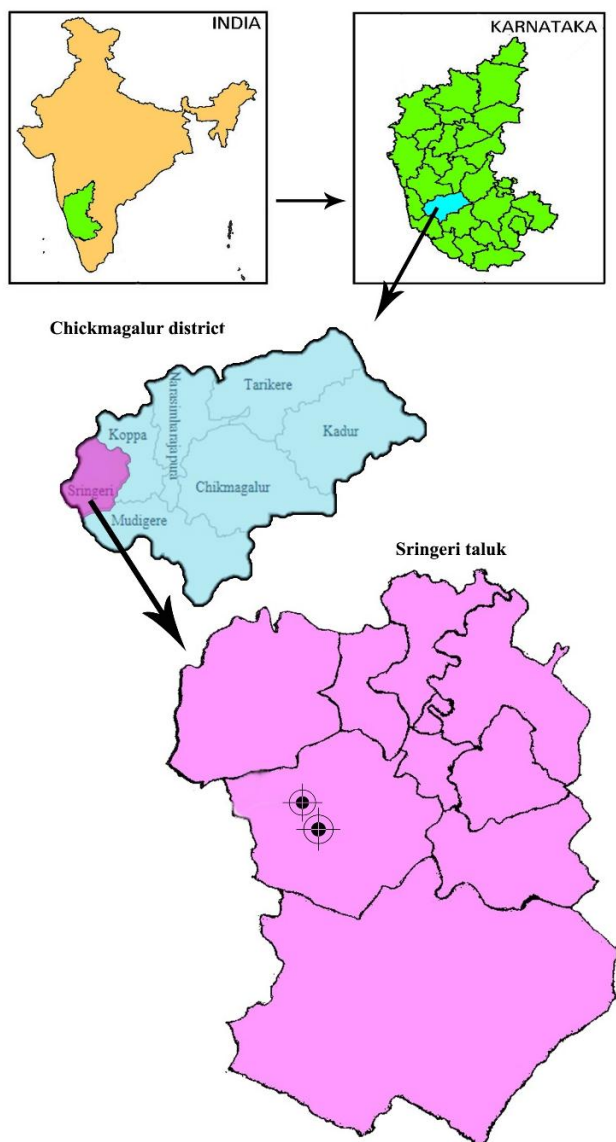


Fig 1: Showing the study sites and sample collected areas in Chikmagalur District

Preliminary Qualitative phytochemical Analysis

The preliminary phytochemical group test of solvent extracts of plant materials for the detection of secondary metabolites were performed by the standard methods [10, 25, 15]. The tests were based on the visual observation of colour change or formation of a precipitate after the addition of specific reagents.

Test for alkaloids

- 1. Mayer's test:** A few drops of the Mayer's reagents was treated with 2 mg of extracts. Formation of white or pale yellow precipitate indicates the presence of alkaloids.
- 2. Wagner's test:** One ml of each extract was mixed with equal volumes of Wagner's reagent (Iodine in potassium iodide). Formation of reddish brown precipitate indicates the presence of alkaloids.
- 3. Dragendorff's test:** 2 mg of the methanolic extract was added with 5 ml of distilled water. To the same 2M hydrochloric acid was added until an acid reaction occurs.

Then 1 ml of Dragendorff's reagent was added. Formation of orange red precipitate indicates the presence of alkaloids.

Test for flavonoids

- 1. Ferric chloride test:** Test solution with few drops of ferric chloride solution shows intense green colour indicates the presence of flavonoids.
- 2. Shinoda test:** To the test solution add few fragments of Magnesium ribbon was added and later, to this add few drops of Conc. hydrochloric acid was also added. Formation of Magenta red colour indicates the presence of flavonoids.
- 3. Zinc-Hydrochloric acid reduction test:** To the test solution pinch of Zinc dust was added later with the addition of few drops of Conc. Hydrochloric acid. Formation of Magenta red colour indicates the presence of flavonoids.
- 4. Alkaline reagent test:** Test solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid.
- 5. Lead acetate solution test:** To the test solution few drops of 10% lead acetate was added. Formation of yellow precipitate indicates the presence of flavonoids.

Test for glycosides

- 1. Keller-Killiani test:** The test solution was treated with few drops of ferric chloride solution and mixed. When concentrated sulphuric acid containing ferric chloride solution was added, it forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green.
- 2. Legal test:** To the test solution few drops of pyridine (made alkali by adding sodium nitroprusside solution) was added. Formation of pink to red colour indicates the presence of glycosides.

Test for tannins

- 1. Ferric chloride test:** To the test solution few drops of ferric chloride solution was added. Formation of dark red colour indicates the presence of tannins.
- 2. Gelatin test:** Test solution when treated with gelatin solution gives white precipitate, it indicates the presence of tannins.

Test for phenols

- 1. Phenol test:** 2 ml of the extracts were separately treated with 1ml of ferric chloride solution. Development of an intense colour indicates the presence of phenols.
- 2. Ellagic acid test:** The extracts were treated with few drops of 5% (w/v) glacial acetic acid followed by 5% (w/v) sodium nitrate solution. Formation of muddy brown colour indicates the presence of phenols.

Test for saponins

- 1. Foam test:** 5 ml of each extract is taken in a test tube and shaken vigorously to obtain a stable froth. To this frothing solution, 5-6 drops of olive oil was added. Formation of an emulsion indicates the presence of saponins.

Test for steroids

- 1. Salkowaski test:** To 1-2 ml of all the extracts, 5 ml of chloroform was added. To the above mixture, 1ml of Conc. Sulphuric acid was added carefully along the walls of the tube and mixed. The formation of reddish color in the lower layer indicates the presence of steroids.
- 2. Liebermann – Burchard’s test:** To 1-2 ml of all the extracts, a few drops of acetic anhydride solution was added. To this mixture, a few drops of Conc. Sulphuric acid was added carefully along the walls of the test tube. Formation of reddish brown ring at the junction of two layers indicates the presence of steroids.

Test for triterpenoids

- 1. Salkowaski test:** Few drops of Conc. Sulphuric acid was added to the test solution, shaken and allowed to stand, lower layer turns yellow indicating the presence of triterpenoids.

Determination of antimicrobial activity**Test microorganisms**

The detection of antimicrobial properties of various extracts against some human pathogenic bacteria, including two Gram – positive bacteria : *Staphylococcus aureus* (MTCC 3160), *Streptomyces pneumoniae* (MTCC 4734); seven Gram – negative bacteria: *Escherichia coli* (MTCC 1559), *Klebsiella pneumonia* (MTCC 2113), *Pseudomonas aeruginosa* (MTCC 1034), *Salmonella typhi* (MTCC 734), *Xanthomonas campestris* (MTCC 2286), *Pseudomonas syringae* (MTCC 1604), *Agrobacterium tumefaciens* (MTCC 431) and the four dermatophytic fungi were *Candida albicans* (MTCC 1637), *Chrysosporium keratinophilum* (MTCC 1367), *Chrysosporium merdarium* (MTCC 4608) and *Trichophyton rubrum* (MTCC 3272). All these microorganisms were procured from IMTECH, Chandigarh, India.

Preparation of inoculums

The bacterial inocula were revived by transferring loop full of organisms from stock culture into a 250ml conical flasks containing sterilized Nutrient broth (Hi Media). The flasks were incubated on a rotary shaker for 24 h at 37°C. The broth cultures were subjected to standard plate count method to enumerate the population and the dilution having 1×10^6

cfu/ml were selected for antimicrobial assay. For fungal inoculums sterilized Potato dextrose broth (Hi Media) was used and incubated for 24 h at 28°C. Using sterile saline solution, the cultures were harvested and diluted to bring the count to about 1×10^8 cfu/ml. Standard Ciprofloxacin and Fluconazole at the concentration 1 mg/ml was used as positive control (standard) against bacteria and fungi respectively and 10% DMSO as negative control. Standard drug solutions were prepared in sterile water for injection.

Antimicrobial screening by agar well diffusion method

The antimicrobial activities of the different extract preparations from the moss *Pogonatum microstomum* was determined by standard agar well diffusion method [21] with slight modifications. Mueller Hinton Agar (Hi Media) and Sabour dextrose agar (Hi Media) plates were swabbed (sterile cotton swabs) with 24 h old-broth culture of respective bacteria and fungi. Then, aseptically, wells of 6 mm diameter were bored in the inoculation plates, with the help of sterile cork borer. To the wells 100 µl of different concentration of (100, 50 and 25 mg/ml of 10% DMSO), standard Ciprofloxacin and Fluconazole, 1 mg/ml and control 10 % DMSO were added separately into the respective labelled wells. The plates were incubated at 37°C for 18- 24 h for bacteria and 28°C for 72 h for fungi in an upright position and the zone of inhibition formed around the wells was recorded. Triplicates were maintained and the experiment was repeated thrice, for each replicates the reading were taken in three different fixed directions and the average values were recorded [27].

Statistical analysis

The experimental results were expressed as mean \pm standard deviation of mean of three replicates. The results were processed using Microsoft Excel 2010.

Results and Discussion**Phytochemical analysis**

The preliminary qualitative phytochemical screening of *Pogonatum microstomum* showed the presence of flavonoids, glycosides, triterpenoids, phenols and sterols are present in all three extracts. Saponins present only in chloroform extract (Table 1).

Table 1: Preliminary screening of secondary metabolites of different extracts of *Pogonatum microstomum*.

Test for Secondary metabolites		Different extracts of <i>P. microstomum</i>		
		Petroleum ether	Chloroform	Methanol
Alkaloids	Mayer’s test	-	-	-
	Wagner’s test	-	-	-
	Dragendroff’s test	-	-	-
Flavonoids	Shinoda’s test	-	-	+
	Zinc-hydrochloride acid test	-	-	-
	Alkaline reagent test	+	+	-
	Ferric chloride test	-	-	-
	Lead acetate solution test	+	-	-
Phenol	Phenol test	+	+	+
	Ellagic acid test	+	+	+
Saponins	Foam test	-	+	-

Tannins	Ferric chloride test	-	-	-
	Gelatin test	+	-	-
Steroids	Salkowaski's test	-	+	+
	Liebermann- Burchardt's test	+	+	+
Glycosides	Keller-Killiani test	+	+	+
	Legal's test	+	-	+
Triterpenoids	Salkowaski's test	+	+	+

Note: '+' - Present, '-' - Absent

Antibacterial activity

The organic solvent (Pet ether, chloroform and methanol) extracts of *Pogonatum microstomum* are subjected to the antibacterial activity against human and plant pathogenic bacteria both Gram-positive and Gram-negative bacteria are presented.

The antibacterial activity of *Pogonatum microstomum* extract shows varied result in different concentration. Among the bacteria tested, *Streptomyces pneumoniae* and *Klebsiella pneumonia* showed the higher degree of inhibition zone (18 mm) followed by *Escherichia coli* (17 mm), *Pseudomonas aeruginosa* (16 mm), *Staphylococcus aureus* (16 mm) and

Agrobacterium tumefaciens (16 mm) in the chloroform extract. Whereas in methanol extract, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* (12 mm) showed the maximum inhibition zone followed by the *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Pseudomonas syringae* (11 mm). In petroleum ether extract, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* showed the maximum inhibition zone (12 mm) followed by *Klebsiella pneumonia*, *Streptomyces pneumoniae*, *Xanthomonas campestris* (11 mm) *Escherichia coli* and *Staphylococcus aureus* (10 mm) (Table 2 and Figure 2, 3 & 4).

Table 2: Antibacterial activity of *Pogonatum microstomum*.

MO	Zone of inhibition (mm)										
	Pet ether			Chloroform			Methanol			Cont.	Std.
	100%	50%	25%	100%	50%	25%	100%	50%	25%		
AT	12 ± 0.25	7 ± 0.58	6 ± 0.13	16 ± 0.14	14 ± 0.25	12 ± 0.19	12 ± 0.28	10 ± 0.44	8 ± 0.26	-	21 ± 0.50
EC	10 ± 0.43	9 ± 0.58	7 ± 0.25	17 ± 0.20	15 ± 0.14	13 ± 0.09	11 ± 0.20	9 ± 0.63	8 ± 0.10	-	15 ± 0.58
KP	11 ± 0.58	7 ± 0.29	-	18 ± 0.29	15 ± 0.43	12 ± 0.09	10 ± 0.13	9 ± 0.29	8 ± 0.58	-	21 ± 0.5
PA	12 ± 0.29	8 ± 0.64	-	16 ± 0.77	13 ± 0.43	11 ± 0.14	12 ± 0.50	10 ± 0.72	8 ± 0.24	-	23 ± 0.76
PS	9 ± 0.29	-	-	14 ± 0.25	12 ± 0.43	11 ± 0.29	11 ± 0.77	9 ± 0.20	-	-	22 ± 0.96
SA	10 ± 0.58	7 ± 0.38	-	16 ± 0.14	14 ± 0.49	12 ± 0.29	11 ± 0.30	9 ± 0.52	-	-	17 ± 0.58
SP	11 ± 0.29	7 ± 0.25	-	18 ± 0.29	16 ± 0.66	14 ± 0.72	10 ± 0.63	8 ± 0.06	7 ± 0.09	-	23 ± 0.50
ST	9 ± 0.58	7 ± 0.25	-	14 ± 0.29	12 ± 0.14	11 ± 0.09	11 ± 0.25	10 ± 0.72	9 ± 0.58	-	14 ± 0.58
XC	11 ± 0.50	8 ± 0.58	6 ± 0.14	14 ± 0.25	12 ± 0.72	11 ± 0.06	10 ± 0.49	9 ± 0.43	8 ± 0.29	-	19 ± 0.76

Note: Std= Standard as Ciprofloxacin (1mg/ml) and Control as 10% DMSO (Growth of organism in the plate is 9 cm), AT- *Agrobacterium tumefaciens*, EC - *Escherichia coli*, KP - *Klebsiella pneumonia*, PA - *Pseudomonas aeruginosa*, PS - *Pseudomonas syringae*, SA - *Staphylococcus aureus*, SP- *Streptomyces pneumoniae*, ST - *Salmonella typhi*, XC- *Xanthomonas campestris*.

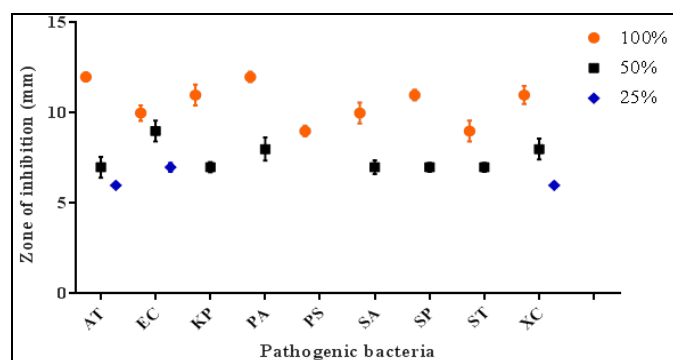


Fig 2: Antibacterial activity of Petroleum ether extracts of *P. microstomum*.

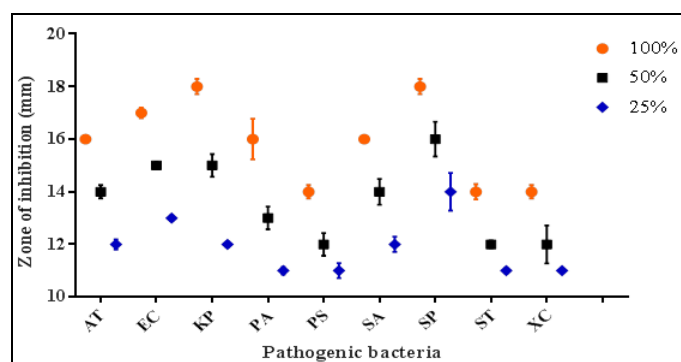


Fig 3: Antibacterial activity of Chloroform extracts of *P. microstomum*.

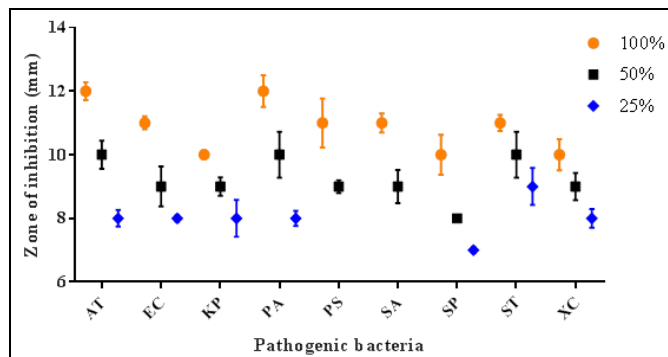


Fig 4: Antibacterial activity of Methanol extracts of *P. microstomum*.

Antifungal activity

The antifungal activity of *Pogonatum microstomum* extract shows varied result in different concentration. Among the fungi tested, against *Candida albicans* showed the higher degree of inhibition zone (13 mm) followed by *Trichophyton rubrum* (8 mm) in the pet ether extract. Whereas in chloroform extract, against *T. rubrum* (10 mm) showed the maximum inhibition zone followed by the *C. albicans* (8 mm). The methanol extract has not shown inhibition zone against all four tested fungi (Table 3).

Banerjee and Sen (1979) had conducted antibiotic activity of 52 species of the bryophytes extracts of water, methanol, ethanol, ether and acetone and tested against 12 microorganisms, including three Gram-positive, five Gram-negative, one acid-fast bacterium and three fungi. Out of 52 species of bryophytes, 29 were active against at least one of the bacteria, but none possessed any antifungal property. In the present study, all extracts showed good active against all nine bacteria and two fungi. But, none possessed any activity against two fungi (*Chrysosporium keratinophilum* and *C. merdarium*). Compare to standard (Ciprofloxacin), Chloroform extract of *P. microstomum* (100% Conc.) showed significant and more or less equal antibacterial property against all tested bacteria [5]. Singh *et al.* (2006) performed an antimicrobial activity of *Plagiochasma appendiculatum* (Aytoniaceae). The plant extract showed significant antibacterial and antifungal activity against the test organisms and also the result showed that *P. appendiculatum* extract has potent wound healing capacity [26]. Kumar and Chaudhary (2010) analyzed the antibacterial activity of crude extract with solvents such as ethanol, petroleum ether, acetone and benzene of *Entodon myurus*, against some pathogenic bacteria. The maximum antibacterial activity showed by acetone and methanol extract against *Enterobacter aerogenes*

while minimum antibacterial activity showed by acetone extract against *Klebsiella pneumonia* [16]. From all these results and review of earlier work, bryophytes have potential activity against human pathogenic bacteria and dermatophytic fungi. *Polytrichum* and *Sphagnum* exhibit strong antibacterial properties against *Gaffkeya tetragena* and *Staphylococcus aureus* [1]. Zhu *et al.* (2006) reasons that minute sizes, difficulties identifying species, and a lack of financial encouragements have hindered efforts to study bryophytes as antimicrobial agents [28].

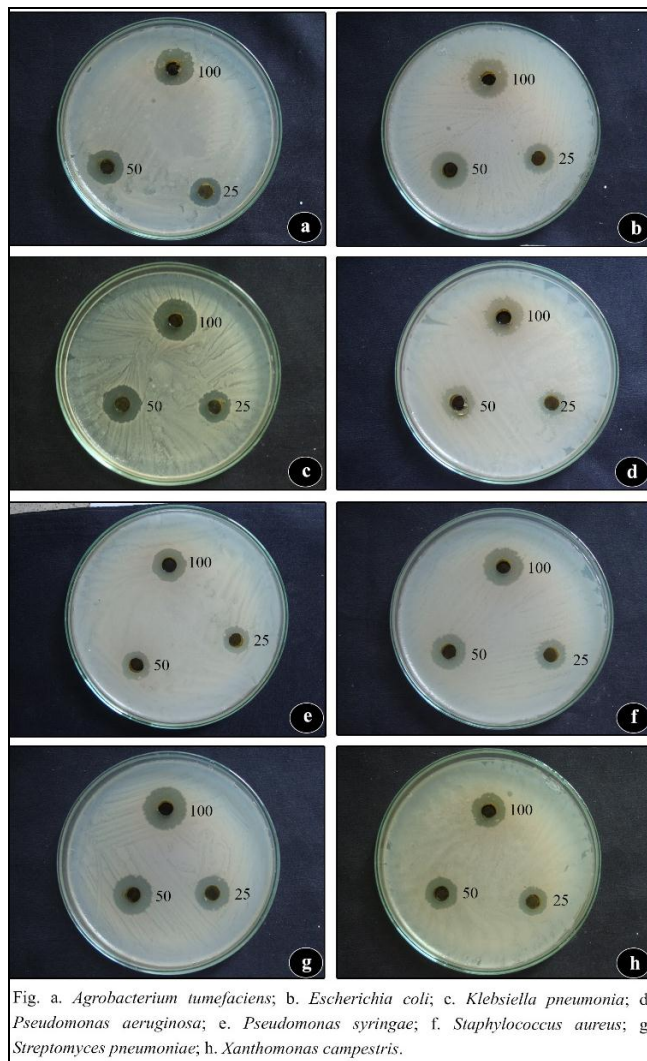


Fig. a. *Agrobacterium tumefaciens*; b. *Escherichia coli*; c. *Klebsiella pneumonia*; d. *Pseudomonas aeruginosa*; e. *Pseudomonas syringae*; f. *Staphylococcus aureus*; g. *Streptomyces pneumoniae*; h. *Xanthomonas campestris*.

Fig 5: Antibacterial activity of Chloroform extracts of *Pogonatum microstomum*

Table 3: Antifungal activities of *P. microstomum*.

Species name	Organism	Zone of inhibition (mm)									
		Pet ether			Chloroform			Methanol			
		100	50	25	100	50	25	100	50	25	Std.
<i>P. microstomum</i>	CA	13±0.25	9±0.38	-	8±0.14	6±0.29	-	-	-	-	24±0.58
	CK	-	-	-	-	-	-	-	-	-	18±0.38
	CM	-	-	-	-	-	-	-	-	-	17±0.96
	TR	8±0.38	6±0.14	-	10±0.25	6±0.43	-	-	-	-	20±0.25

Note: Std= Standard as Fluconazole (1mg/ml) and Control as 10% DMSO (Growth of organism in the plate is 9 cm), CA- *Candida albicans*, CK- *Chrysosporium keratinophilum*, CM- *Chrysosporium merdarium* and TR- *Trichophyton rubrum*.

Conclusion

The bryophytes are delicate land plants and are lacking of thick cuticle and bark therefore they have biochemically active compounds which perform defense mechanism to protect them from enemies like fungi, bacteria and insects. In the present study *Pogonatum microstomum* was showed the presence of useful secondary phytochemical constituents and it showed good antibacterial and antifungal activities.

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