Research Article

Comparison of antimicrobial potentiality of the purified terpenoids from two moss species *Thuidium tamariscellum* (C. Muell.) Bosch. & Sande-Lac and *Brachythecium buchananii* (Hook.)A. Jaeger

Abstract

Microbicidal resistant pathogens have increased tremendously; meanwhile the search for the new antibiotics has reduced. Herbals are treasure islands of many phytochemicals with potential biological features. Bryophytes are the oldest lineage of land plants and mostly unexplored in terms of their medicinal importance. This study targets to extract terpenoids, its purification, fractionation and evaluation of microbicidal potentialities from *Thuidium tamariscellum* and *Brachythecium buchananii* against *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *Streptococcus mutans* and *Bacillus cereus*. Fungal species includes *Aspergillus flavus* and *Candida albicans*. The analysis include susceptibility determination test, effect of the extract on cell membrane integrity and fungal spore inhibition assay. It was found that the *T. tamariscellum* terpenoid extract was more effective than the *B. buchananii* extract. *T. tamariscellum* terpenoid extract displayed the significant minimum inhibitory concentration and minimum killing concentration i.e., the values ranged from 0.0625 to 0.125mg/mL and 0.25 to 0.5mg/mL against *S. aureus* and *E. coli* respectively. However, both the moss species showed remarkable antifungal property. Nucleic acid, protein leakage and relative electrolyte conductive % substantiate the MIC and MKC values of the bacterial strains. Spore inhibition value increases in proportion to concentration of the extracts which further confirms the fungicidal power. Overall, the terpenoid extract from *T. tamariscellum* has potent antimicrobial activity and may be a potential source of lead molecules for the future development of microbicidal agents. Further studies are warranted to analyze the morphological deformities using electron microscopy.

Keywords: bryophytes, antimicrobial, inhibitory concentration, killing concentration, membrane permeability, spore, terpenoids

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Introduction

Mosses are small nonvascular spore bearing land plants distributed throughout the temperate and tropical parts of the world. There are approximately 14,500 species which constitutes around 75% among the bryophyte species. They are commonly found in moist shady locations. The species originated as early as the Permian period and more than 100 species have been recorded from fossils of the Paleogene and Neogene periods.[1] Mosses perform various important ecological roles such as filtering and retaining water, stabilizing the ground and removing CO_2 from the atmosphere. Many moss species were reported to possess unique natural products or secondary metabolites such as phenols, flavonoids, alkaloids, terpenoids and other aromatic compounds with therapeutic potentialities. Historically, the therapeutic features of herbals are the concept of doctrine of signatures. Bryophytes form the basement of Chinese medicinal treatment. For example, liverwort was used to cure hepatic disorders, *Polytrichum commune* induce women's hair growth. Gaddi tribes of Himachal Pradesh, used *Plagiochasma appendiculatum* for treating skin diseases, *Targionia hypophylla* used by Irular tribec of Attappady to cure skin diseases due to resembles of thallus to the warty surface of the diseased region and *Frullania ericoides,* liverwort for hair-related applications by tribal people of South India. Species like *Sphagnum*, *Barbula*, *Bryum*, *Octeblepharum* and *Fontinalis* are used to treat different diseases, including cardiovascular diseases, inflammation, fever, lung diseases, infections, wounds and skin prone diseases.[1] The aqueous extract of the three mosses like *Brachythecium rutabulum, Calliergonella cuspidate* and *Hypnum mammillatum* showed potent antioxidant activity.[2,3] The species like *Polytrichum commune* were used as antipyretic and anti-inflammatory agent and boiled with tea for treating the cold. *Rhodobryum giganteum* is another species traditionally used to treat diseases like cardiovascular diseases or angina.[1]

The moss *Plagiochilla beddomei* possesses significant antioxidant activity.[4] The moss *Physcomitrella patens* under axenic condition produces a tetracyclic diterpene, namely 16α–hydroxykaurane (16α-hydroxy-ent-kaurane, Kaurenol, $C_{20}H_{34}O$.[1] α and β pininealloromadendrine from *Plagiochilla stevensoniana* were useful as anticancer and antimicrobial compounds.[5] Mosses retard the growth of cancer cells in *in vitro* culture studies. The plant derived natural products occupy an important place in the area of cancer chemotherapy because of minimal side effects. *Polytrichum commune* plays significant role especially for the therapy of lymphocytic leukemia.[5]

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Phenolic compounds isolated from *Atrichum*, *Dicranum*, *Mnium*, *Polytrichum* and *Sphagnum* spp. were reported to possess antimicrobial properties. The antimicrobial activity for three moss species *Eurhynchium angustirete*, *Rhytidia delphussquarrosus* and *Rhodo bryumroseum* was validated by Nikolajeva.[6] *Thuidium gratum, Ectropothecium aeruginosum, Sematophyllum caespitosum, Stereophyllum radiculosum, Babulalam berenensis, Campilopusa spericuspis* and *Calympereserosumlam berenensis, Campilopusa spericuspis* and *Calympereserosum* were proved for their potential antimicrobial properties.[7] The moss *Atricum undulatum* possesses strong antifungal activity against *Aspergillus versicolor* and *A. fumigates*.[8]

The outbreak of multiple antibiotic tolerant Gram-positive, Gram-negative bacteria and fungal pathogens in hospitals and community centers requires new approaches for fast diagnosis and treatment. Prioritization of infectious agents for research and development is need of the hour that defines the impact of pathogens on human health. Ranking antibiotic-resistant organisms in order to direct research and development requires an accurate identification and integration of mass knowledge that explains the issue of antimicrobial resistance organisms in terms of microbiological, epidemiological and clinical ways.

Terpenoids group of isoprenoids are diverse group of natural products derived from the five carbon isoprene units. Based on the number of isoprene units they are classified into hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenids (C25) and triterpenoids (C30). Terpenoids possess a wide range of medicinal properties for the treatment of various illnesses such as cancer, malaria, inflammation and various infectious diseases (viral and bacterial).

Therefore, the present study was aimed to analyze the antimicrobial activities of the purified terpenoid extracts from two moss species *Thuidium tamariscellum* and *Brachythecium buchananii* against Gram positive and Gram negative bacteria and selected fungal strains. Parallely the mode of action of the terpenoid in terms of their microbicidal power was also analyzed.

Materials and methods

Plant materials

Brachythecium buchananii and *Thuidium tamariscellum* were belongs to Brachytheciaceae and Thuidiaceae of Musci. Both the species grow as epiphytes on trunks of trees and also on moist rocky and shady surfaces of high altitude regions. The identity of the two species were confirmed by floras and authenticated by comparing with the herbarium of University of Calicut. Voucher specimens were deposited in the herbarium of Department of Botany, University College, Thiruvananthapuram UBH 630 and UBH 631.

Column chromatography and GC-MS analysis

The crude methanolic extract of the mosses were purified using silica gel column chromatography and eluted with petroleum ether: ethyl acetate as solvent combinations. The different eluted column fractions were subsequently quantified for the presence of terpenoid as per the method described by Ferugson.[9] The purified fraction was subjected to GC-MS analysis. The chromatogram obtained containing a pool of compounds and the mass spectrum of the unknown components were compared with the spectrum of the known components available with the NIIST library.

Antibacterial assay

Test microorganisms: *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeroginosa* (ATCC 27853), *Streptococcus mutans* (MTCC 890) and *Bacillus subtilius* (ATCC 6633).

Petriplates containing 20 ml Muller Hinton agar medium were seeded with bacterial culture of *E. coli, Pseudomonas aeroginosa, Streptococcus mutans, Bacillus subtilius* and *Staphylococcus aureus* (growth of culture adjusted according to McFards Standard, 0.5%. Wells of approximately 10mm was bored using a well cutter and different concentrations of terpenoid extracts such as 0.25mg/mL, 0.5mg/mL, 0.75mg/mL, 1.0mg/mL 1.5mg/mL and 2.0mg/mL were added. The plates were then incubated at 37°C for 24h. The bactericidal activity was assayed by measuring the diameter of the inhibition zone (mm) formed around the well.[10] Streptomycin was used as a positive control.[10]

Antifungal assay

Test organisms were *Aspergillus flavus* (ATCC 16404) and *Candida albicans* (ATCC 10231)**.** Freshly prepared potato dextrose agar (PDA) plates and were swabbed with overnight grown fungal species such as *Aspergillus flavus and Candida albicans*. Wells of approximately 10mm was bored using a well cutter and different concentrations of terpenoid extracts such as 0.25mg/mL, 0.5mg/mL, 1.0mg/mL 1.5mg/mL and 2.0mg/mL were added. The zone of inhibition was measured after overnight incubation at room temperature and compared with that of standard antimycotic clotrimazole.[10]

Minimal inhibitory concentration (MIC) was determined by using two-fold serial dilution method of Eloff[11] with some modifications. Briefly, the suspensions of bacterial strains prepared from overnight broth cultures, were adjusted to the required microbial density (about 10^7 CFU/mL). Terpenoid extracts was dissolved in DMSO, then twofold serial dilutions were made in a concentration ranging from 0.0625 to 2.5mg/mL in 10mL sterile test tubes. A 50μL suspension of the strains was added into the tube. The tube containing only broth and the strain was the negative control. The MIC was determined as the lowest concentration of the extract that demonstrated no visible growth in tubes after 24h.

Minimal killing concentration (MKC) was analyzed by sub-culturing the different doses that had no visible growth. 100μL from each dilution was inoculated on the surface of freshly made nutrient agar plates and incubated for 24h at 37°C. The minimum concentration that had no detectable growth on agar media after 24h incubation was noted as MKC.

Permeability of bacterial membrane

The bacterial membrane permeability was expressed in the relative electric conductivity according to the protocol of Diao et al.[12] The bacterial strains was separated by centrifuging at 5,000×*g* for 10min, and then washed with 5% glucose until the electric conductivity was approximately near to that of 5% glucose, which suggests the case of isotonic bacteria. The electric conductivity was recorded by an electrical conductivity meter. Terpenoid extracts at three different

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doses (control, 1×MIC, and 2×MIC) were added into 5% glucose; the electric conductivity of the mixtures was measured and marked as L_1. Then different doses of terpenoid extracts (control, 1×MIC, and 2×MIC) were added into the isotonic bacteria, respectively. After completely mixing, the samples were incubated at 37°C for 10h; the conductivity was measured per 2h; it was marked as L_2. The conductivity of bacteria in 5% glucose treated in boiling water for 5 min was used as the control which was marked as L_0. The permeability of cell membrane is quantified using

Relative electric conductivity (%) = $100 \times (L_2 - L_1)/L_0$

Bacterial membrane integrity

The bacterial cell membrane integrity was analyzed by the protocol of Du et al.,[13] with marginal modifications. 100mL of working culture of bacteria was centrifuged at 3000°g for 15min. The bacterial cells were collected, washed thrice and re-suspended in 0.1M pH 7.4 phosphate buffer solutions. 100mL buffer treated bacterial culture was incubated with different doses of terpenoid extracts (control, MIC and 2×MIC) at 37°C for 6 h under agitation. Then 25mL of the aliquots were collected and centrifuged at 11,000×g for 5min. The protein content in the supernatants was quantified by the Bradford method.[14]

Analysis on nucleic acids leakage

Propidium iodide (PI) is a dye that binds effectively to nucleic acids. The dye is not capable of intrude in to viable cells suggesting it as a marker to analyze the effects of drugs on bacterial membranes leakage. The selected bacterial cells were suspended in 0.9% saline medium (OD_{600}=1.5). The suspensions were further exposed to terpenoid extracts at doses of the MIC and × 2 MIC in duplicate for 10 min. The bacteria (1mL) were centrifuged for 1 min at 11000×g. The pellet was washed with 1mL 0.9% saline solution. 3μl of PI was added to each sample and the solution was mixed thoroughly. The samples were kept in the dark for 10 min. Fluorescence was recorded at excitation and emission at 544nm and 612nm, respectively, using an *f*max microplate spectrofluorometer. The controls used were non treated cells, 3% DMSO and streptomycin.

Effect of terpenoid on spore germination

The effect of different doses of the terpenoid extracts of *B. buchananii* and *T. tamariscellum* on spore germination of *C. albicans* and *A. flavus* was studied. 1μl of each extract at different doses (0.125mg/mL, 0.25mg/mL, 0.5mg/mL and 1.0mg/mL) was poured into 50ml conical flasks and 9ml of the PDA was added to each conical flask which gave PDA-extract mixture with corresponding extract concentration. These doses were used to study spore germination of the isolates. Spore suspensions of *C. albicans* and *A. flavus* containing 20-30 spores per microscopic field was prepared from 7 days old cultures of isolates. One drop of spore suspension was put in a glass slides containing a drop of different doses of terpenoid extract. Hence slides were kept in moist chamber prepared by putting two folds of filter paper in both side of the Petri dishes were incubated at 28⁰C for 24h.[15] The spore germination % was recorded by the method of Salhi et al.[16]

$$Percent\ spore\ germination = \frac{Number\ of\ spore\ germinated}{Total\ number\ of\ spores} \times 100$$

Statistical analysis

Statistical analyses were attempted using One-Way analysis of variance (ANOVA) of SAS 8.0 software to analyze the difference of DIZ, MIC, MKC, relative electric conductivity, the concentration of leaching nucleic acids, proteins, spore inhibition among control, 1×MIC and 2×MIC groups. The statistically significant level was set as 0.05.

Results and discussion

GC-MS analysis of the moss species

The 100% PE column eluted fraction of *T. tamariscellum* and *Brachythecium buchananii* showed a single band on TLC. GC-MS analysis yielded 15 terpenoids in *T. tamariscellum* such as α-cubebene, caryophyllene, 1-tetradecene, dodecanal, tetradecane, heptadecane, pentadecane, hexadecane, eicosane,octadecane, nonadecane, heneicosane, docosane, pentacosane and hexacosanes. Similarly, GC-MS analysis of *Brachythecium buchananii* displayed 5 compounds such as hexadecane, hexadecanoic acid, octadecane, heneicosane and tetracosane.

Martins et al.,[17] reported that the essential oil of *Cordia* species showed a pool of terpenoids including caryophyllene and was effective against the treatment of buccal and inflammatory pathogens. The petroleum ether extract of *Citrus medica* seeds an ethanomedicinal plant was used for the treatment of piles, inflammation showed the presence of n-hexadecanoicacid, heneicosane, nonadecane and tetracosane.[18].

Antimicrobial activity

Microbiologists documented the efficacy of herbal extracts and their phytochemicals as microbicidal agents to inhibit growth of bacteria. Some phytochemists have recorded that microbicidal agents like terpenoid of phenolic group interact with enzymes and biomolecules of the microbial cell membrane causing its derailment to disperse a flux of protons towards cell exterior which induces cell death or block enzymes necessary for metabolism.

The antimicrobial activity of various doses (0.25mg/mL, 0.5mg/mL, 0.75mg/mL, 1.0mg/mL, 1.5mg/mL 2.0mg/mL) of terpenoid extracts of *B. buchananii* and *T. tamariscellum* against *E. coli, Pseudomonas aeroginosa, Streptococcus mutans, Bacillus subtilius* and *Staphylococcus aureus* were qualitatively and quantitatively analyzed by the presence of inhibition zones. As presented in **Table 1,** the diameter of inhibitory zone (DIZ in mm) values for all the strains increased significantly ($P < 0.05$) along with the increasing concentration of the extract. Remarkable inhibitory zone values were noticed with *Staphylococcus aureus* followed by *E. coli* i.e., 26.1±0.65 and 17.3±0.16mm respectively treated with terpenoid extracts of *T. tamariscellum*. However, *B. buchananii* also showed a more or less similar trend but with lesser values. Most of the strains were susceptible against the terpenoids except *Streptococcus mutans* and *Bacillus subtilius* (resistant forms).

Laura et al.[19] demonstrated that the moss species *Climacium dendroide* and *Polytrichum commune* possess strong antibacterial activity against the bacterial strains such as *Bacillus cereus, Staphylococcus aureus* and *E. coli. Hylocomium splendens* and *Pleurozium schreberi* showed significant antibacterial activity against

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Pseudomonas aeruginosa (inhibition zone diameter 12 mm).[20] Pejin et al.,[20] reported that *Climacium dendroides*, *Ptilium crista-castrensis*, *Rhytidiadelphus triquetrus* and *Sphagnum magellanicum* showed strong antibacterial activity against *E. coli*.

Meanwhile, the fungal species such as *A. flavus* and *C. albicans* were significantly inhibited by the terpenoid extracts of *T. tamariscellum* (25.9±0.84 and 22.5±0.48 respectively) and *B. buchananii* (22.5±0.85 and 20.1±0.04 respectively) The values were comparable with the synthetic antibiotics streptomycin and clotrimazole. The MIC values for terpenoid extracts from *T. tamariscellum* were 0.0625 and 0.25mg/mL for *Staphylococcus aureus* and *E. coli* respectively. In the case of *B. buchananii* the respective values were 0.125±0.03 and 0.5±0.06 (Table 2).

Weerakkody et al.[21] commented the mode of classification of microbicidal power into strong activity i.e., DIZ >20mm, (moderate = <20mm), and weak = (DIZ <12mm). Similarly, Aligiannis et al.[22] suggested that MIC value up to 0.5µl/mL as strong; moderate if MIC 0.6–1.5µl/mL, and low above 1.5µl/mL. The results of the present study showed that terpenoid extracts displayed strong to moderate antimicrobial activity against the selected strains. It could be attributed to the major components like α-cubebene, caryophyllene, 1-tetradecene, dodecanal, tetradecane, heptadecane, pentadecane, hexadecane, eicosane, octadecane, nonadecane, heneicosane, docosane, pentacosane and hexacosanes which seems to increase the permeability of cell membrane or disintegrate the outer membrane of Gram-negative bacteria. MICs of fungal species *A. flavus* were 0.0625±0.09 and 0.125±0.0234 against *T. tamariscellum* and *B. buchananii* respectively. The entire spectra of MICs substantiate the zone of inhibition data of the respective strains (Table 2).

Table 1 Microbicidal activity of purified terpenoid from *Thuidium tamariscellum* and *Brachythecium buchananii* determined by agar diffusion assay. Mean±SD, *p*<0.05

Conc. (mg/ml) T. tamariscellum	E. coli	P. aeruginosa	S. mutans	S. aureus	B. subtilis	A. flavus	C. albicans
0.25	3.55±0.03	4.4±0.02	1.4±0.08	8.5±0.09	0.94±0.07	7.2±0.03	6.8±0.51
0.5	5.4±0.02	6.2±0.09	2.5±0.08	11.2±0.05	1.5±0.21	9.5±0.20	10.2±0.4
0.75	8.9±0.02	7.1±0.04	4.6±0.11	14.5±0. 4	2.2±0.23	11.3±0.05	12±0.02
1.0	12.5±0.05	8.5±0. 5	6.3±0.35	20.6±0.47	4.3±0.04	17.3±0.26	15.4±0.06
1.5	15.4±0.32	10.6±0.12	8.9±0.09	24.7±1.25	5.9±0.01	23.8±0.22	19.8±0.67
2.0	17.3±0.16	12.6±0.21	10.3±0.08	26.1±0.65	7.6±0.01	25.9±0.84	22.5±0.48
Conc. (mg/ml) B. buchananii	E. coli	P. aeruginosa	S. mutans	S. aureus	B. subtilis	A. flavus	C. albicans
0.25	2.8±0.07	2.7±0.09	0.4±0.01	8±0.02	0.35±0.09	7.4±0.03	6.7±0.07
0.5	4.4±0.01	3.8±0.05	0.95±0.04	10±0.2	2.0±0.11	9±0.08	8±0.05
0.75	6.5±0.06	5.3±0.01	2.0±0.08	13±0. 08	3.2±0.03	10.7±0.2	9.4±0.04
1.0	10.8±0.03	9±0.22	4.8±0.07	17.6±0.34	4.3±0.4	14±0.1	12.9±0.02
1.5	13.1±0.05	12.1±0.04	7±0.02	20±0.54	5.9±0.1	19.6±0.05	18±0.07
2.0	15.4±0.07	14.1±0.33	8.8±0.021	22.4±1.54	7.6±0.05	22.5±0.85	20.1±0.04

Table 2 MIC and MKC of purified terpenoid of Thuidium tamariscellum and Brachythecium buchananii (mg/ml). Mean±SD, p<0.05

Organism	Thuidium tamariscellum		Brachythecium buchananii	
	MIC	MKC	MIC	MKC
E. coli (-)	0.25±0.04	0.5±0.05	0.5±0.06	1.0±0.02
P. aeruginosa (-)	0. 50±0.13	1.0±0.07	0.75±0.03	1.50±0.01
S. mutans (+)	2.0±0.49	4.0±0.08	2.5±0.11	5.0±0.25
S. aureus (+)	0.0625±0.01	0.125±0.01	0.125±0.03	0.250±0.02
B. subtilis (+)	2.5±0.09	5.0±0.07	0.75±0.07	1.5±0.056
A. flavus	0.0625±0.09	0.125±0.09	0.125±0.0234	0.25±0.08
C. albicans	0.125±0.01	0.25±0.03	0.25±0.022	0. 5±0.045

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MKC of *T. tamariscellum* and *B. buchananii* were in the range of 0.125–5.0mg/mL and 0.25–5mg/mL respectively, against all tested strains (Table 2). MIC values of terpenoid extracts were significantly ($p < 0.05$). Similarly the MKCs of *C. albicans* were 0.25 ± 0.03 and 0.5 ± 0.045 against *T. tamariscellum* and *B. buchananii* respectively. The values were significant at 5% level.

Irfan et al.[23] reported that terpenoids from *Sphaeranthus indicus* L showed strong antimicrobial activity against *Escherichia coli* and *Klebsiella pneumonia*. The terpenoids such as beta-sitosterol, alpha-amyrin, lupeol, hexacosanoic acid, ceryl alcohol and hexacosane from *Trichodeama amplexicaule* possess potent microbicial activity against *Escherichia coli* and *A. flavus*.[2]

Mode of antibacterial mechanism

The antibacterial activity of terpenoid extract may be through affecting the external membrane and the cytoplasm. The hydrophobicity of the terpenoid components enables them partition in the lipid fractions of the membranes and also disrupting the mitochondrial structures, altering their functions from permeably.[25] This in turn, derails the production of biomolecules like DNA, RNA, protein which leads to the death of the cells.[26] The GC-MS analysis of the terpenoid extracts of the species may attributes the specific mode via disrupting the targets in the cell.[27]

Cell membrane permeability

Cell membrane potential remarkably regulates the microbial balance and resistance to antibiotics.[28] Normal physiological conditions of bacterial cells have a negative charge due to the presence of anionic entities like carboxyl and phosphate in the membrane.[29] Drugs depolarize the membrane pay way for reduction of the membrane potential volume. In the present results, the PI intensity may be directly correlated with the membrane potential. The loss of cell membrane depolarization of bacteria cells may leads to irregular cell metabolic activity and there by death of the bacterial cells.

Nucleic acids leakage

The fluorescence of propidium iodide (PI) in the bacterial cells after exposure to the *T. tamariscellum* and *B. buchananii* extracts was shown in the Figure 1. Against *S. aureus*, the fluorescence of PI significantly enhanced with the dose of the terpenoid extract. The *T. tamariscellum* extract caused the high nucleic acids leakage for *S. aureus* i.e., at the MIC (0.0625mg/mL) and double the MIC (0.125mg/mL), the fluorescence of PI was 2.33 fluorescence units (F/units) and 3.02 F/units, respectively. The fluorescence of PI was lowest in the *B. subtilis* cells, which was 0.29 and 0.94 F/units at MIC and ×2 MIC. In *E. coli*, following exposure to the extract, the fluorescence of PI was 1.54F/units and 2.5F/units after exposure to the MIC and ×2 MIC respectively. The *B. buchananii* extracts at MIC and double the MIC caused nucleic acid leakage 1.12 and 2F/units in *E. coli* cells. However, terpenoid extracts showed less efficacy in terms of fluorescence of PI with *S. mutans*. Untreated cells showed a negligible PI fluorescence of F/units. The same was true for cells treated with DMSO also.

Protein leakage

B. subtilis and *S. mutans* cells displayed insignificant protein leakage with both the moss species (Table 3). Meanwhile, the terpenoid extracts treated strains such as *E. coli, Pseudomonas aeroginosa,* and *Staphylococcus aureus* lost more protein as compared to control cells. The *T. tamariscellum* extract at the MIC and double the MIC caused significantly more protein leakage in *Staphylococcus aureus* than the untreated cells. Streptomycinin caused the leakage of high amounts of protein. In the cells treated with DMSO the leakage was marginal i.e., 0.007mg/mL. These amounts were similar to the untreated cells i.e., 0.0052mg/mL of protein was leaked from the cells. The *B. buchananii* extract caused protein leakage at double the MIC was 0.026mg/mL against *Staphylococcus aureus*. The protein leakage observed in the untreated cells was insignificant.

Polyphenols are powerful molecules of disrupting the integrity of the cell wall/membrane like lipopeptides bind to bacteria and cause rapid depolarisation of the membrane and eventual bacterial cell death.[30] *T. tamariscellum* extract was capable of causing damage to the *S. aureus* and *E. coli* membranes resulting in nucleic acid and protein leakage (Figure 1) (Table 3). DMSO did not cause any damage to the membranes as the protein and nucleic acid leakage from the bacterial cells was less. Although the *B. buchananii* extract caused leakage of nucleic acids and protein in *E. coli*, the leakage was less compared to *T. tamariscellum*.
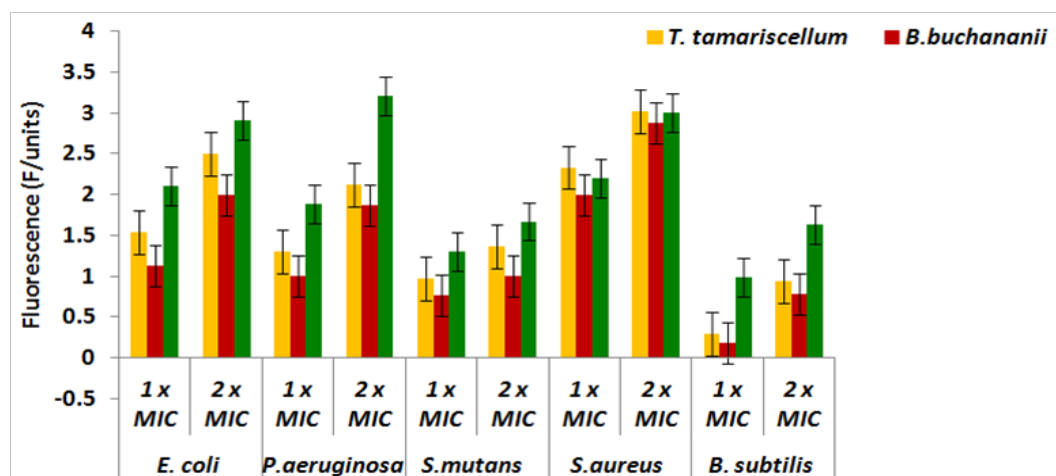

Figure 1 Fluorescence of PI leakage against selected bacterial strains with *T. tamariscellum* and *B. buchananii* terpenoid extracts.

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Table 3 Protein leakage (mg/ml) against selected bacterial strains with *T. tamariscellum* and *B. buchananii* terpenoid extracts

	E. coli		P. aeruginosa		S. mutans		S. aureus		B. subtilis	
	1xMIC	2xMIC	1xMIC	2 x MIC	1 x MIC	2xMIC	1xMIC	2xMIC	1xMIC	2MIC
T. tamariscellum	0.023	0.031	0.02	0.028	0.014	0.021	0.03	0.048	0.009	0.012
B. buchananii	0.019	0.025	0.015	0.02	0.01	0.018	0.026	0.037	0.005	0.009
Streptomycin	0.025	0.036	0.024	0.03	0.018	0.025	0.032	0.0489	0.01	0.019

Relative electric conductivity of bacterial membrane

Cell membrane permeability was evaluated in terms of relative electric conductivity. The % of relative electric conductivity of cells treated with terpenoid extracts demonstrated that *T. tamariscellum* extracts have a positive effect on the membrane permeability of *S. aureus* and *E. coli*. The terpenoid extracts resulted in increased relative electrical conductivity of bacterial cells, which indicated a leakage of intracellular ingredients especially electrolytes from the cells. The maximum relative permeability were 69% and 62% at concentration of 2×MIC for *S. aureus* and *E. coli* respectively, that was more than *B. buchananii* terpenoid extracts as shown in the Table 4. Regulating ion homeostasis is essential to the optimal balance of energy level, transport of solutes, regulation of metabolism, turgor pressure control and cell motility, thus a marginal change of the integrity of the membrane structure may influence metabolism and lead to death of the cell. The biomolecules like nucleic acids and proteins were leached from the treated bacteria, an indicator of bacterial cell membrane integrity in comparison to control cells. The overall results of the study suggest the non-recoverable cytoplasmic membranes damage.

Thus, cell membrane integrity was considered as an critical factor to inhibit the growth of bacteria. Further research is warranted to analyze the targeted sites on bacteria cells to make sure that either bactericidal effect was from damage to lipopolysaccharide or membrane proteins in cell wall. Saritha et al.[31] analyzed the antibacterial mode of action of the alcoholic extracts of *Hemidesmus indicus Leucas aspera*, *Plumbago zeylanica* and *Tridax procumbens*. Pai-Wei Su et al.,[32] displayed similar antibacterial activities mechanism of *Polygonum cuspidatum* extracts against Nosocomial Drug-Resistant Pathogens. Marasini et al.,[33] evaluated antibacterial activity of ethnic medicinal plants against human pathogenic bacteria. Vandal et al.,[34] screened antimicrobial activity of natural products from the flora of Northern Ontario, Canada. Aboshora et al.,[35] studied the effect of extraction method and solvent power on polyphenol and flavonoid levels in *Hyphaene thebaica* as antibacterial. Fernando et al.,[36] viewed mode of antimicrobial nanoparticles and mechanisms of action. Wang et al.,[37] role of nanoparticles as antimicrobial agent was confirmed. Bilal Sadiq et al.,[38] proved membrane instability was the major reason of antibacterial activities of *Acacia nilotica* against Multidrug-Resistant bacteria. Diao et al.[12] analyzed the chemical composition, antibacterial activity and mode of action of essential oil from seeds of fennel. Bajpai et al.,[39] explained antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens.

Sadiq et al.,[40] screened the phytochemicals and *in vitro* antibacterial activities of leaves, pods and bark extracts of *Acacia nilotica*. Borges et al.,[28] proved antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Zhao et al.,[41] confirmed antibiotic activity and mechanisms of sugarcane extract against food-borne pathogens. Qader et al.,[42] explained antibacterial activity of plant extracts against clinical pathogens. Mahboubi et al.,[43] isolated phenolic and flavonoid content of *Punica granatum* flower and validated bactericidal power against bacterial strains causing food borne diseases.

Sonam et al,.[44] demonstrated that nanoparticles (NPs) functionalized with essential oils have significant antimicrobial potential against multidrug- resistant pathogens due to an increase in chemical stability and solubility, decreased rapid evaporation and minimized degradation of active essential oil components.

Vidal et al.,[45] screened the secondary metabolites and its synergistic action from the bryophyte *Octoblepharum albidum*. Bhattarai et al.,[46] proved antioxidant activity of *Sanionia uncinata*, the artic moss from King George Island, Antarctica. Alam[47] reviewed Indian bryophytes and their biologically active compounds. Singh et al.,[48] confirmed antimicrobial activity of some Indian mosses. Khanam et al.,[49] validated antibacterial activity of *Marchantia palmata*. Asakawa[50] summarized the advances in phytochemistry of bryophytes-acetogenins, terpenoids and bis(bibenzyl)s from selected Japanese, Taiwanese, New Zealand, Argentinean and European liverworts. Xie and Lou[51] screened phytochemicals in bryophytes: an ecological aspect. Bodade et al.,[52] attempted *in vitro* screening of bryophytes for antimicrobial Activity. Hahn et al.,[53] isolated biaurone, a triflavone and biflavonoids from two *Aulacomnium* species. Basile et al.,[54] detected antibacterial activity of pure flavonoids isolated from selected mosses. Mukhopadhyay et al.,[55] screened the antimicrobial and antioxidative potential of selected eastern himalayan mosses. Many antibiotics such as oxytetracycline and chlortetracycline, streptomycin, sulfonamides, tylosin, erythromycin, chloramphenicol, and lincomycin are used to control bacterial pathogens worldwide. However, many reports have showed the development of resistant strains in the isolates. Bryophytes chemically synthesize wide terpenoids, phenolics as microbicidal against fungi and prokaryotes. In this study, the microbicidal activities of bryophytes were tested for the first time in the literature against Gram positive, Gram negative and fungal species. The terpenoids of these bryophytes may be used as an alternative to the regular antibiotics. All the above works substantiates the microbicidal potential of the moss species obtained in the present study.

Fungal spore inhibition assay

Figure 2 shows the effect of *T. tamariscellum* and *B. buchananii* terpenoid extracts on spore germination against fungal pathogens like *C. albicans* and *A. flavus*. Both the extracts showed significant

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inhibition against the two tested fungi. Spore germination of *C. albicans* and *A. flavus* was inhibited to 76 and 72% by *T. tamariscellum* extract. Similarly, *B. buchananii* terpenoid extract was also equally effective. For pathogenic fungi, spore germination is an optimal factor at the onset of their colonization, and the tested extracts have a definite potential, which can be used as a new potential fungicide. The assay values further substantiates the zone of inhibition, MIC and MKC values. Most Ferdousi Begum et al.,[56] analyzed spore and mycelial growth inhibition of three fruit rot pathogens using some synthetic fungicides and botanical extracts. Silva et al.,[57] studied fungicidal activity of medicinal plant extracts against pathogens of coffee. Awad and Baka[58] reported the herbal extract control of the fungi associated with different Egyptian wheat cultivars grains.

Table 4 Relative electric conductivity (%) against selected bacterial strains with *T. tamariscellum* and *B. buchananii* terpenoid extracts

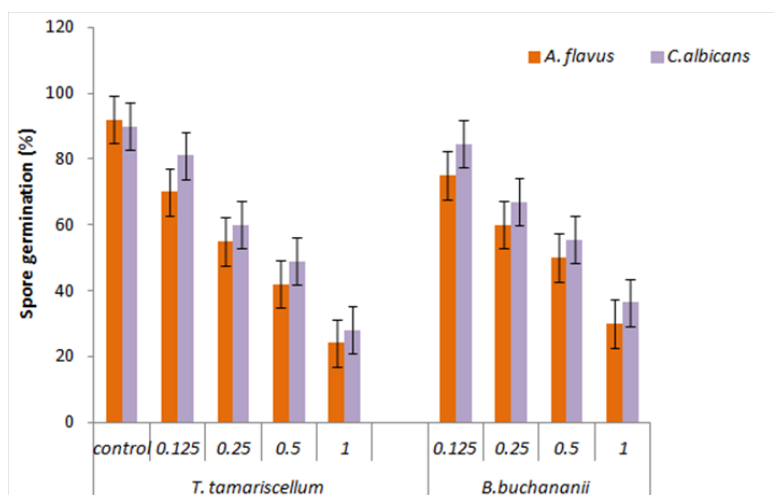|	*T. tamariscellum*									
	E. coli		*P. aeruginosa*		*S. mutans*		*S. aureus*		*B. subtilis*	
	1x MIC	2x MIC	1xMIC	2xMIC	1xMIC	2x MIC	1x MIC	2x MIC	1xMIC	2MIC
0	0	0	0	0	0	0	0	0	0	0
2	14	23.1	10	14.3	7	9	18.7	20	3	5
4	29.4	36.3	23.5	26.2	13	18.6	32	37	7	11
6	42.3	48.4	36.1	41	19.8	22	46	50	9.4	13
8	54	60	42	46.8	29	31	62	67	13	16
10	56	62	48	50	31	34	64	69	18	20
	B. buchananii									
0	0	0	0	0	0	0	0	0	0	0
2	10	13	7.3	10	4	6	16	18	2	3
4	21	25	15	17.5	9	13	30	33	5	7
6	33	36	24	30	13	17	40	45	8	10
8	42	47	30	37.8	20	24	52	56	11	13
10	44	45.8	32	40	22	28	54	57.4	15	19
	Streptomycin									
0	0	0	0	0	0	0	0	0	0	0
2	17	25	13	16	10	13	24	28	9	12
4	31	39	26	30	18	21	37	43	14	20
6	44	50	39	47	27	30	50	60	21	28
8	58	64	46	55	38.5	45	66	70	30	38
10	61	66	50	60	40	50	70	72	33	41

Figure 2 Spore inhibition assay against selected fungal strains using *T. tamariscellum* and *B. buchananii* terpenoid extracts.

Comparison of antimicrobial potentiality of the purified terpenoids from two moss species Thuidium tamariscellum (C. Muell.) Bosch. & Sande-Lac and Brachythecium buchananii (Hook.)A. Jaeger

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Conclusion

Based on the present research, the *T. tamariscellum* and *B. buchananii* terpenoid extracts displayed a optimal microbicidal activity against *S. aureus* and *E. coli*. Terpenoid extracts caused the alterations in the cell wall and membrane of the pathogens. As per the results, the mode of action against *S. aureus* and *E. coli* may be membrane break through the permeability of cell membrane, and then led to the leakage of electrolytes, proteins and DNA. These changes resulted in to cell death eventually, which were corresponded to a simultaneous reduction in the number of viable cells. However, because of the heterogeneous compositions, it seems unlikely that there is more than one mode of action responsible for the antimicrobial action. Therefore, further research is warranted to understand the modes, such as the inhibition of pathogens, the interactions with other ingredients in order to justify the real applications of terpenoid in practices as a natural microbicidal agent. Bryophyte species are unexplored and may be an alternative effective approach because of their safety, relatively low cost and effectiveness against pathogens.

Acknowledgements

Conflict of interest

The author declares that there is no conflict of interest.

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