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 unplored 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 Strepctococcus 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 terpneoid 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 confirmsthe 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 microbiological agents. Further studies are warranted to analyze the morphological deformities using electron microscopy.

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

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 CO₂ 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, Polytichium commune induce women’s hair growth. Gaddi tribes of Himachal Pradesh, used Plagiochasma appendiculatum for treating skin diseases, Targionia hypophylla used by Iruaral tribe of Attapady to cure skin diseases due to resembles of thallus to the warty surface of the diseased region and Frullania ericoide, liverwort for hair-related applications by tribal people of South India. Species like Sphagnum, Barbula, Bryum, Octoblepharum 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 cuspidata and Hypnum mammillatum showed potent antioxidant activity.2,3 The species like Polytichium 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-hydroxykauran (16α-hydroxy-ent-kaurane, Kaurenol, C₂₉H₄₆O₃, a and β pininealloromadendrine from Plagiochilla stevensoniana were useful as anticancer and antimicrobial compounds.5 Mosses retard the growth of cancer cells in vitro culture studies. The plant derived natural products occupy an important place in the area of cancer chemotherapy because of minimal side effects. Polytichium commune plays significant role especially for the therapy of lymphocytic leukemia.5

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Phenolic compounds isolated from *Atrichum*, *Dichranum*, *Mnium*, *Polycirrhum* and *Sphagnum* spp. were reported to possess antimicrobial properties. The antimicrobial activity for three moss species *Eurhynchium angustirete*, *Rhytidia delphussquarrosus* and *Rhodobryumroseum* was validated by Nikolajeva.\(^6\) *Thuidium gratum*, *Ectropothecium aeruginosum*, *Senophyllum caespitosum*, *Streophyllum radiculsum*, *Babululum berenensis*, *Campilopusa spericuspis* and *Calymperesosserum* 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), sesterterpenoids (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 microbialidal 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 NIST library.

### Antibacterial assay

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

Petriplates containing 20 ml Muller Hinton agar medium were seeded with bacterial culture of *E. coli*, *Pseudomonas aeroginosa*, *Streptococcus mutans*, *Bacillus subtilis* 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.

### 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 and 2.0mg/mL were added. The zone of inhibition was measured after overnight incubation at room temperature and compared with that of standard antifungal clotrimazol.\(^10\)

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 Dio\(^12\) and 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 L0. 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 10 h; the conductivity was measured per 2 h; it was marked as Lp. The conductivity of bacteria in 5% glucose treated in boiling water for 5 min was used as the control which was marked as Lc. The permeability of cell membrane is quantified using

Relative electric conductivity (%) = 100 × (Lp - Lc)/Lc

**Bacterial membrane integrity**

The bacterial cell membrane integrity was analyzed by the protocol of Du et al., with marginal modifications. 100 mL of working culture of bacteria was centrifuged at 3000 g for 15 min. The bacterial cells were collected, washed thrice and re-suspended in 0.1 M pH 7.4 phosphate buffer solutions. 100 mL 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 25 mL of the aliquots were collected and centrifuged at 11,000 g for 5 min. The protein content in the supernatants was quantified by the Bradford method.

**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 (OD400 = 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 (1 mL) were centrifuged for 1 min at 11,000 g. The pellet was washed with 1 mL 0.9% saline solution. 3 μL 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 544 nm and 612 nm, respectively, using an fmax 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. 1 μL of each extract at different doses (0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL) was poured into 50 ml conical flasks and 9 ml 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 slide containing a drop of different doses of terpenoid extract. Hence slides were kept in moist chamber prepared by putting two folds of slides containing a drop of different doses of terpenoid extract. 10 min. The bacteria (1 mL) were centrifuged for 1 min at 11000×g. The selected bacterial cells were suspended in 0.9% saline solution. 3 μL 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 544 nm and 612 nm, respectively, using an fmax microplate spectrofluorometer. The controls used were non treated cells, 3% DMSO and streptomycin.

**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, hexadecane, eicosane, octadecane, nonadecane, 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., 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 ethnomedicinal plant was used for the treatment of piles, inflammation showed the presence of n-hexadecanoic acid, heneicosane, nonadecane and tetracosane.

**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 microbial 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 antagonistic activity of various doses (0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL) of terpenoid extracts of B. buchananii and T. tamariscellum against E. coli, Pseudomonas aeruginosa, Streptococcus mutans, Bacillus subtilis 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±0.16 mm 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 subtilis (resistant forms).

Laura et al. demonstrated that the moss species Climacium dendroides and Polystichum commune possess strong antibacterial activity against the bacterial strains such as Bacillus cereus, Staphylococcus aureus and E. coli. Hylomicum splendens and Pleurozium schreberi showed significant antibacterial activity against

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Table 1 Microbicidal activity of purified terpenoid from Thuidium tamariscellum and Brachythecium buchananii determined by agar diffusion assays. 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         | 3.5±0.03        | 4.4±0.02 | 1.4±0.08      | 8.5±0.09  | 0.9±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±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 |
|--------------|------------------------|--------------------------|
| E. coli (-)  | 0.25±0.04              | 0.5±0.05                 |
| P. aeruginosa (-) | 0.5±0.13          | 1.0±0.07                  | 0.75±0.03  | 1.5±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±0.023| 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 hexacosanone from *Trichodeama amplexicaule* possess potent microbicial activity against *Escherichia coli* and *A. flavus.\(^3\)

**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 aeruginosa*, 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. Streptomycin 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](image-url) 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 1xMIC | E. coli 2xMIC | P. aeruginosa 1xMIC | P. aeruginosa 2xMIC | S. mutans 1xMIC | S. mutans 2xMIC | S. aureus 1xMIC | S. aureus 2xMIC | B. subtilis 1xMIC | B. subtilis 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. 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. displayed similar antibacterial activities mechanism of Polygonum cuspidatum extracts against Nosocomial Drug-Resistant Pathogens. Marasini et al. evaluated antibacterial activity of ethnic medicinal plants against human pathogenic bacteria. Vandal et al. screened antimicrobial activity of natural products from the flora of Northern Ontario, Canada. Aboshora et al. studied the effect of extraction method and solvent power on polyphenol and flavonoid levels in Hyphaene thebaica as antibacterial. Fernando et al. viewed mode of antimicrobial nanoparticles and mechanisms of action. Wang et al. role of nanoparticles as antimicrobial agent was confirmed. Bilal Sadig et al. proved membrane instability was the major reason of antibacterial activities of Acacia nilotica against Multidrug-Resistant bacteria. Diaol analyzed the chemical composition, antibacterial activity and mode of action of essential oil from seeds of fennel. Bajpai et al. explained antibacterial mode of action of Curcuma tricispidata fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens.

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

Sonam et al. demonstrated 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. screened the secondary metabolites and its synergistic action from the bryophyte Octoblepharum albidum. Bhattachari et al. proved antioxidant activity of Sanionia uncinata, the arctic moss from King George Island, Antarctica. Alam reviewed Indian bryophytes and their biologically active compounds. Singh et al. confirmed antimicrobial activity of some Indian mosses. Khanam et al. validated antibacterial activity of Marchantia palmata. Asakawa summarized the advances in phytochemistry of bryophytes-acetogenins, terpenoids and bis(bibenzyl)ys from selected Japanese, Taiwanese, New Zealand, Argentinean and European liverworts. Xie and Lou screened phytochemicals in bryophytes: an ecological aspect. Bodeade et al. attempted in vitro screening of bryophytes for antimicrobial Activity. Hahn et al. isolated biaurone, a triflavone and biflavonoids from two Aulacomnium species. Basile et al. detected antibacterial activity of pure flavonoids isolated from selected mosses. Mukhopadhyay et al. screened the antimicrobial and antioxidant potential of selected eastern himalayan mosses. Many antibiotics such as oxytetracycline and chlorotetracycline, 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 microbial against fungi and prokaryotes. In this study, the microbial 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 microbial 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. Table 4 Relative electric conductivity (%) against selected bacterial strains with T. tamariscellum and B. buchananii terpenoid extracts.

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

|          | E. coli | P. aeruginosa | S. mutans | S. aureus | B. subtilis |
|----------|---------|---------------|-----------|-----------|-------------|
|          | 1x MIC  | 2x MIC        | 1xMIC     | 2xMIC     | 1x MIC      | 2x MIC      | 1xMIC | 2xMIC |
| 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    |

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

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**Conclusion**

Based on the present research, the *T. tamariscellum* and *B. buchananii* terpenoid extracts displayed a optimal microbial 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.

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**Conflict of interest**

The author declares that there is no conflict of interest.

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