International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Original Article **CODEN: IJPNL6**

BACTERICIDAL POTENTIALITY OF FLAVONOIDS EXTRACTED FROM CELL SUSPENSION CULTURES OF *MARCHANTIA LINEARIS* LEHM & LINDENB.

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Received on: 23-01-2016; Revised on: 13-03-2016; Accepted on: 24-03-2016

ABSTRACT

The present investigation was undertaken to isolate and fractionate flavonoids from *in vitro* cell culture of the liverwort *Marchantia linearis* Lehm & Lindenb., a poorly documented bryophyte and its mode of bactericidal potentiality. Initially, callus culture was initiated from spores in MS/5 media containing growth regulators. From suspension culture flavonoids were isolated and fractionated by HPLC PAD chromatogram, which revealed the presence of a pool of active compounds. *In vitro* time-kill assessment data supports the MBC and MKC. Remarkable leakage of reducing sugar and high leakage of nucleic acid and protein were observed at different concentration of flavonoid. Further, respiratory chain enzyme dehydrogenases showed lower profile in the treated bacterial strains. SEM data substantiated the bactericidal potentiality. Thus, the flavonoids of *M. linearis* may be a potent candidate for the *in vivo* biological control of pathogenic bacteria. Further studies are warranted to purify the lead molecule and to elucidate the molecular mechanism of bactericidal action.

Keywords: Antibacterial activity, Bryophytes, Cell suspension, Flavonoids, *Marchantia linearis*.

INTRODUCTION

Bryophyte, the simplest, primitive non-vascular plants represents the second largest group in the plant kingdom. Screening of biologically potential phytochemicals is an important aspect in the field of pharmacology. Many studies have reported the presence of remarkable amounts of polyphenols, steroids, glycosides and tannins in bryophyte [1]. Diverse flavonoids are reported from mosses and its biosynthesis is catalyzed by chalcone synthase (CHS). The entire enzymatic pathways of flavonoid synthesis from bryophytes are still unknown. The cloning and characterization of chalcone synthase (CHS) from the moss, *Physcomitrella patens* was reported by Jiang *et al.* [2]. Antibiotic like compounds were also reported from these groups [3].

Traditional Indian medicine with its progress through years has always fascinated practitioners and researchers in terms of curing diseases through herbals on a scientifically proven research background. Systematic study of plants and understanding their active principles is worth towards pharmaceutical industries in order to develop magic drugs for curing various diseases.

Currently, tissue culture has become an essential tool in supplying stable research materials for both chemists and botanists. In this juncture, the present study is an attempt to elucidate the flavonoids from the *in vitro* cultures of *Marchantia linearis* Lehm & Lindenb. and evaluate their antibacterial potentialities.

MATERIALS AND METHODS

Plant material: *Marchantia linearis,* thalloid liverwort forms dichotomously branched thalli (Figure 1). They were collected from the lower Kallar river floor of Ponmudi hills, Trivandrum, Kerala. Taxonomic identity was confirmed by comparing

with liverwort flora and authenticated by comparing herbarium specimen at Department of Botany Herbaria, University of Calicut, Kozhikode (UC 029).

Microorganisms: Gram positive bacteria viz., *Staphylococcus aureus* (NCIM-2079), *Bacillus subtilis* (NCIM-2712), *B. cereus* (NCIM-2016) and Gram negative bacteria includes *Salmonella typhimurium* (NCIM-2501), *Proteus vulgaris* (NCIM-2168), *Escherichia coli* (NCIM-2089), *Klebsiella pneumoniae* (NCIM -2957) and *Pseudomonas aeruginosa* (NCIM-2200) were obtained from National Collection of Industrial Micro-organisms (NCIM), NCL, Pune.

Establishment of cell suspension culture: Mature spores from sporangia were selected as explants and they were disinfected with propamidine and pentamisine (5 µg/mL) and surface sterilized with 10% sodium hypochlorite + two drops of Tween- 20. The media employed were Knops, Gamborg, Murashiq skoog and 1/5 diluted MS medium supplemented with antibiotics. After germination, the effective explants were transferred onto different multiplication media, calli multiplying vigorously were transferred on to differentiation media of MS/5 with varying concentrations of NaH_2PO_4 (100 to 250 mg/L) containing growth regulators BAP (2 mg/L) and NAA (0.5 mg/L).

To establish suspension cultures, 2-14 g green friable calli were aseptically transferred to MSK-2 liquid medium supplemented with varying growth regulators on a rotary shaker at 110 rpm [4]. Cells were continuously sub-cultured every 3 weeks at 23ºC. Productivity (in mg/L/day) was calculated as the final yield of flavonoid (mg/L) divided by the total culture period (in days). Flavonoid productivity was optimized by standardizing various culture parameters like carbon source, growth regulators, light intensity, inoculum size, cations environmental stress and elicitors. Culturing was terminated as soon as the glucose in the medium was exhausted. Culture experiments were carried out in triplicates.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) PAD analysis of flavonoids: Ethyl acetate-methanol (1:3) eluted flavonoid extract was subjected to RP-HPLC PAD analysis. The chromatographic system (Waters Company) consisted of Millennium 32 system software, Waters 717 plus Auto sampler, Model Waters Delta 600 pump, and Model Waters 2996 Photodiode Array Detector. The chromatographic peaks of the analytes were confirmed by comparing their retention times and UV spectra with those of the reference standards. Quantification was carried out by integration of the peak using external standard method [5].

Microbicidal potentials: All the selected bacterial stock cultures (section 4.1.3) were maintained at 4°C on nutrient agar medium (Hi-Media). Active cultures for experiments were prepared by transferring a loopfull of culture to 10 mL of nutrient broth Muller Hinton Agar (MHA) (Hi-Media) and incubated at 37°C for bacterial proliferation. The bacterial suspension after incubation (24h) was extracted by centrifugation at 9000g for 15 minutes. The resulted pellet was dissolved in distilled water and the quantity was made to 1 x 10^8CFU /mL by absorbance at A_{610} nm).

Microbicidal assay by disc diffusion method: Fresh petriplates were prepared with 20 ml of sterile Mueller-Hinton Agar (Hi-media). The solidified media was swabbed on top with the respective test cultures and dried for 10 min. The *in vitro* potential of flavonoids of *M. linearis* as microbicidal agents was carried out by disc diffusion method [6]. The results were compared with that of Ampicillin the antibiotic and DMSO served as positive control.

Assay of Minimum inhibitory concentration (MIC) by ELISA: Micro dilution method was used to determine the MIC of the flavonoid extract. Briefly 4-5 isolated colonies were selected from an overnight bacterial culture and were diluted in broth to turbidity comparable to that of a 0.5 McFarland turbidity standard (approximately 1.0×10^8 CFU/mL). The microtiter plates were incubated at 38±2 ℃ for 1 day. 1h before the end of incubation, 40 µL of a 0.2% solution of Iodo-Nitro Tetrazolium (INT) (Merck, Germany) was added to the wells and the plate was incubated for another hour. The assay was repeated thrice. After the incubation period, the plates were read at 620 nm using ELISA reader.

Minimum Killing Concentrations: Different concentrations of the flavonoid extract prepared were 0.0625, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 mg/mL. To determine minimum killing concentration, 50 µL broth was taken from each well and inoculated in 200 µL nutrient broth for 24 h at 37°C for bacteria cells. Minimum killing concentration is defined as the lowest concentration of the extract at which inoculated microorganism was completely killed. Each test was performed in triplicate and repeated twice.

Time kill assay (Survival-time study): Mid-logarithmic phase cultures of selected bacterial

strains were taken in pre-warmed Mueller-Hinton Agar nutrient broth (50 mL of Hi-Media make, containing flavonoid extracts in concentrations of ½ x MIC, 1 x MIC and 2 x MIC to get a final concentration of 10^5 CFU/mL. The broths were maintained on a water bath at 30°C, with gentle agitation. From this, 1 mL were drawn at regular intervals (0, 6, 12, 24, 30 and 36 h) and aptly diluted with agar medium to balance the impact of *M. linearis* flavonoid extracts and viable counts were evaluated by pour plate methods. The experiments were performed thrice.

Cell membrane leakage assay: The method of Karsha and Lakshmi [7] was followed to determine the leakage of UV_{260} and UV_{280} absorbing material.

Effect on the sugar leakage of membrane from the bacteria: A volume of 10 ml culture containing 10^8 CFU/mL of the pathogenic bacteria were inoculated into 50, 100, 200, 400, 500 µg/mL flavonoid extracts of *M. linearis* and were incubated at 37± 2ºC, shaken at 150 rpm for 12/24 h. The samples were centrifuged at 12,000 rpm and the supernatant were collected and frozen at -20ºC. The concentration of reducing sugar was determined using 3-5 Dinitro salicylic acid (DNS) reagent at wave length 550 nm [8].

Assay of respiratory chain dehydrogenase enzyme activity in the bacteria: Dehydrogenase activity assay was done using 2,3,5- triphenyl tetrazolium chloride (TTC) as the artificial electron acceptor, which is reduced to red coloured triphenyl formazan (TPF). The assays were done in 3 ml volumes of nutrient broth-glucose-TTC medium, supplemented with varying concentrations of *M. linearis* flavonoid extracts in 20 ml screw-capped test tubes. The TPF produced after each exposure period were extracted in 4 mL of amyl alcohol and determined spectrophotometrically at 500 nm. The amount of formazan produced was determined from a standard dose-response curve (R^2 =0.9999). Data were expressed as the mean and standard error. Dehydrogenase activity was expressed as µg of TPF formed /mg dry cell weight of cell biomass/h [9].

Statistical analysis: Each data point was the mean of three replicates obtained from 3-5 independent experiments. All experimental data were analyzed by an analysis of variance (ANOVA). After confirming the significance of F values, the significance of the differences between the mean values was tested using ANOVA. Significant differences were considered at $P< 0.01$ probability levels.

RESULTS AND DISCUSSION

Cell suspension culture and optimization of flavonoid productivity: 5 g of the friable callus was found optimal to form suspension in 12-14 days from inoculation time in the liquid medium. The inoculum size had a positive effect on biomass and flavonoid production under optimal conditions. Agitation of the suspension at lower rpm brought about lower level of cell dispersion, while at higher rpm results into increased cell collision. Thus, the rpm was kept at optimal speed i.e., 110 rpm. Continuous sub culturing process substantially improves cell growth and biomass. During the initial subculture, cells were seen growing as free, single or joint cells (Figures 2a & b). Generally, aggregation in plant cell culture is desired because metabolite production is not always the function of a single cell, but involves mass of cells and hence cell aggregation is a favourable property with respect to the production of secondary metabolites [10]. *M. linearis* suspension cultures in the MSK-2 medium showed typical time course of flavonoid synthesis i.e., flavonoid content was at par with cell growth. Maximum flavonoid content was noticed between 12- 16th day of culture.

Cell suspension cultures of *M. linearis* were unable to grow profusely at ≤ 37°C. Culture standardization suggests that 25°C was ideal for flavonoid synthesis. Carbon nutrient source was known to regulate the cell growth, metabolism and yield in *in vitro* culture experiments. Biomass and flavonoid synthesis were maximum with glucose as carbon source compared to sucrose, fructose and galactose. In continuation, the amount of glucose required to produce optimal biomass and flavonoid synthesis were evaluated. At lower glucose concentration the cells are green and viable but showed less growth. Meanwhile, at 2 -3% glucose concentrations, biomass and flavonoid content attained the maximum level suggesting the need of glucose as source of carbon for cell proliferation and secondary metabolism production. Medium supplemented with phytohormones singly or with different combinations were assesed. Highest flavonoid productivity was obtained in cultures with 2,4-D (i.e., 12.8 mg/L/day).

In the present culture experiments, the various light intensities (photon flux) have no remarkable impact on biomass of cells. Inspite, at 20 µmol/m^2/s photon flux flavonoid level reached the peak i.e., 12 mg/L/day The increase in flavonoid yield was mainly due to the induction of secondary metabolic pathway in the cells than growth rate. The effect of different size of the inoculum against the cell growth and flavonoid content was evaluated. Positive correlation was noticed with inoculum size with biomass and flavonoid productivity up to 12% followed by a

decrease No significant changes were noticed in cell growth and flavonoid productivity by the addition of cations such as Mg^{2+}, Mn^{2+}, Cu^{2+} and Ca^{2+} to the standard culture medium as salts of sulphate. However, flavonoid content was enhanced with iron salts (12 mg/L/day). NaCl and sucrose at different concentration inhibit biomass production and which inturn the flavonoid level. Secondary metabolic pathways are usually elicitated by synthetic or natural elicitors. Different concentrations of methyl jasmonate (MJ) and 2-(2 fluro 6-nitrobenzyl sulfanyl) − pyridine 4- carbothioamide were used to enhance flavonoid productivity in *Marchantia* cell line culture. Dose dependent increase was noticed in flavonoid content confirming the efficiency of elicitors in the induction of secondary metabolic pathways. The overall study suggests that flavonoid synthesis was proportional to biomass during the *in vitro* culture period. Flavonoid content and cell growth rate reveals that secondary metabolism in cells was controlled by biomass density.

Quantification of total flavonoids by RP-HPLC PAD: Extraction of flavonoids with 80% ethanol under the frequency of 100 kHz, at 25^0C, the liquid-solid ratio of 10 mL/g with 15 min duration repeated thrice gave the highest flavonoid yield (Figure3). Optimal repeatability, relative standard deviation (RSD) and recovery (97.27 - 99.68%) were noticed for flavonoids with RP-HPLC/PAD analysis. The major flavonoids identified and quantified in *M .linearis* were quercetin (487.65 µg/g), luteolin (587.8 µg/g), apigenin (256 µg/g), rutin (187 µg/g) and Kaempferol (146 µg/g).

Bactericidal potentiality: Flavonoid of *M. linearis* exhibited varied bactericidal potentialities against the bacteria such as *Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Bacillus cereus, B. subtilis, Proteus vulgaris* and *Pseudomonas aeruginosa* at the different concentrations tested. The microbicidal potential of the flavonoid was visualized as inhibition zone by treating the pathogens with the extract and then spreading the cells on agar plates by disc diffusion assay (Figures4 a-e). Among the tested pathogens, *E. coli, S. aureus, Bacillus cereus, B. subtilis* and *K. pneumonia* were the most sensitive and *Pseudomonas aeruginosa, Proteus vulgaris*and *S. typhimurium,* the resistant species with *M. linearis.* The minimum inhibitory concentration (MIC) for the extract of *M. linearis* ranged from 0.06 to 0.62 mg/ml, while minimum bactericidal concentration (MBC) from 0.18 to 1 mg/mL (Table 1). The mechanisms of antibiosis indicated by ampicillin and flavonoid extract were highly significant against the entire tested bacterial isolates, even though, the flavonoid is not as effective as the synthetic antibiotic.

Time-kill curve analysis: In the time-kill curve assay, the results presented in terms of the changes in the $\log_{10}$ CFU/mL of viable colonies indicated that the flavonoid of *M. linearis* exhibited a significant bactericidal activity. After 4 h of incubation the bacteria *S. aureus* with the 1 × MICs and 2 × MICs, the average reduction in the viable cell count ranged from 1.135 to − 0.264 CFU/mL (Table 2). After 8 h of incubation with these concentrations, the reductions in the viable cell count were 0.183 and −2.213 CFU/mL. At 1 × MICs and 2 × MICs, the incubating bacteria *B. subtilis* for 4 h resulted in the reduction of the viable cell count ranging between 1.109 and −0.161 CFU/mL, while at 8 h, the reduction ranged between 0.045 and −3.968 CFU/mL. Meanwhile, at 2 × MICs, incubating the bacteria *K. pneumoniae* for 4 h resulted in the reduction of the viable cell count to 0.562 CFU/mL while after 8 h of incubation, the average reduction in the viable cell count was at −1.313 CFU/mL. Time kill assay data revealed that *Staphylococcus aureus, Bacillus cereus, E. coli* and *B. subtilis* being the significantly sensitive bacteria among the isolates.

Scanning electron microscopic analysis (SEM) of E. coli and S. aureus: SEM images of treated bacterial cells showed different surface anomalies compared with the control. The most striking morphological feature was the concavity on the cell surface, illustrating the abnormality of cell segregation step during cytokinesis. Further, cell deformation such as reduced sizes and shrinkage were also noticed. The abnormalities noticed in the SEM may be due to the action of flavonoids of *M. linearis* on the bacterial cells of *E. coli* and *S. aureus*. It is likely that the flavonoids elicit inhibition in cell division and cell growth, which was expected to be the cause of the early declining phase, together with the induction of membrane dysfunction. Control cells were intact with smooth surface (Figure5a) compared to the treated cells (Figures 5b&c). The other morphometric deformities were shrinking, leakage of cytoplasm, depression of the cell walls with outgrowths and large cell aggregations. Although, the samples were not evaluated quantitatively, it was clearly observed that the number of damaged cells were significantly higher in the treated samples than in the control.

Membrane leakage of bacteria: Membrane leakage of reducing sugars was marginal during the initial period (0 h), in the control bacterial cells. Meanwhile, the leakage amount of reducing sugar was remarkable

in the *M. linearis* flavonoid treated bacterial cells (50 µg/ml) i.e., 6 µg/mg bacterial dry weight in *B. cereus* followed by *B.subtilis* (5.8 µg/mg). After treatment with 500 µg/mL for 24 h, the leakage amount of reducing sugar was up to 18 µg/mg bacterial dry weight in *S. aureus*, but it was only 8.2 µg/mg in *S. typhimurium*, suggesting that flavonoids cause differential damage on the cell membrane of the bacterial strains, which may accelerate the reducing sugar leakage from the cytoplasm. In *B. cereus* and *B. subtilis*, the leakage of sugar after 24 h was 17 µg/mg (Table 3a & b).

Flavonoid extract of Marchantia species on respiratory chain dehydrogenase of bacteria: The effect of *M. linearis* flavonoid on respiratory chain dehydrogenases of the different bacterial cells decreased proportionally with the incubation time and concentration compared to control (Table 4). Interestingly, enzymatic activity of cells treated with 10 µg/mL extract was higher than that of control at 15 min, but its activity reduced drastically with increase of incubating time. Similarly, with 30 µg/mL extract, the enzymatic activity further started decreasing with duration. Significant reduction of enzymatic activity was accounted with 50 µg/ml extract treatment. The results suggest that the activity of respiratory chain dehydrogenases of bacteria could be inhibited by the flavonoids. Among the studied bacteria,*Salmonella typhimurium*,*Proteus vulgaris* and *Pseudomonas aeruginosa* were the most resistant with flavonoids of *M. linearis*.

Leakage of nucleic acid and protein: Effect of the flavonoid of *M. linearis* was also evaluated in terms of leakage of UV_{260} and UV_{280} absorbing material (mainly nucleic acid and protein) through the bacterial cell membrane (Table 5). The leakage of proteins and nucleic acids from cells in control was low, while leakage of these from cells treated with the extract increased significantly. The leakage of proteins and nucleic acid enhanced with duration than in control experiment.

The extensive use of antibiotics in medicinal and agricultural fields has resulted in the emergence of antibiotic-resistant microbes, capable of causing severe life-threatening infections which are hard to manage [11]. These microbes are resistant to multiple classes of antimicrobials i.e. multidrug-resistant microbes. Bryophytes are important source of potentially useful new chemotherapeutic agents. Their usage in traditional medicine as curatives for several diseases is popularized among the world population in Asia, Africa and South America. There has been an increasing interest in the exploration of antibacterial plant products having mechanisms of action different from those of the conventional chemical drugs. Biological activity of polyphenols is multiple, such as permeabilize membrane or potential to form irreversible complex with nucleophilic amino acids in proteins. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes [12]. This aspect is effectively evaluated by flavonoids extracts of *Marchantia* species, on the selected bacterial strains.

The Gram positive bacterium *S. aureus* was significantly inhibited by the flavonoids of *M. linearis* followed by the species viz., *B. subtilis* and *E .coli*. The results are comparable with the antibiotic ampicilin. However, *M. polymorpha* is effective against Gram negative bacteria like *P. aeruginosa* and *P. vulgaris*. This differential resistance of the bacterial species was due to their cell wall chemistry. Cell wall of Gram positive bacteria comprise multiple layers of peptidoglycan (8-30nm thickness) interconnected by side chains and cross bridges. Further, cell wall is also associated with teichoic acid. Contrarily, Gram negative bacteria possess low amount of peptidyl glycan and can be easily disintegrated by bactericidal agents. Gram negative bacteria lack teichoic acid. Peptidoglycan is situated in the periplasm space and covalently linked to lipo-protein in the outer membrane [13]. In addition, unusual barriers of Gram negative bacteria towards antibiotic drugs was due to the rich hydrophilic lipopolysaccharides (LPS) covalently bound to the lipid A of the membrane. This LPS model block the antibacterial drugs. Additionaly, the enzymes in the periplasm space are potential to denature the chemicals which enter into the cell [14].

The highly polar nature of ethanol due to its hydroxyl (OH) group, with the high electronegativity of oxygen, allows hydrogen bonding to take place with other molecules. Ethanol therefore attracts polar and ionic molecules. The ethyl group in ethanol was non-polar and therefore attracts non-polar molecules. Low dielectric constants and dipole nature also add ethanol as an ideal solvent for extracting plant secondary metabolites effectively than other solvents [13]. The type and level of biological activity exhibited by any plant material depends on many factors, including plant part, geographical source, soil condition, drying method and post-harvest processing. The variation in composition of active compounds in flavonoid extract of *Marchantia* species resulted in significant difference ($P < 0.01$) on the level of bactericidal activity (inhibitory zone) against the tested bacterial strains.

The effectiveness of an antibacterial agent wasusually measured by its ability to inhibit and kill bacteria. *In vitro* time-kill assays are expressed as the

rate of killing by a fixed concentration of an antimicrobial agent and is one of the most reliable method for determining tolerance. Generally, the effect of the flavonoids of *Marchantia* species on the tested bacteria in this experiment was time and concentration dependent, as evident from the data presented. At higher concentration (2×MIC) and longer duration of interaction (8 h), more bacteria were killed. The significant reduction in the cell counts between 4 and 8 h of incubation period reveal the fact that the extract is highly bactericidal, suggesting that the bacterial colonies are almost reduced after incubating for 8 h. On the contrary, there is a net growth of all the test isolates treated with the 1/2 × MICs of the extract. While the growth inhibition and efficacy of the flavonoids are dose and time dependent to present effective time-kill profiles for the tested bacteria, the results of the antibacterial assays determined by the agar diffusion as well as the macro broth dilution assay are complementary. These results are further substantiated by the observed rate of time kill assay.

Additionally, the activity of respiratory chain dehydrogenases in the bacteria might be inhibited by the flavonoids significantly at the higher concentration. It is assumed that the flavonoid may break the barrier of outer membrane permeability, peptidoglycan and periplasm, and destroy respiratory chain dehydrogenases, furthermore inhibiting respiration of cells. Rogers and Li [15], Chanderand Brookes [16] reported metal ions inhibited respiration by interacting with thiol (–SH) group of cysteine by replacing the hydrogen atom to form –S–metal, thus hindering the enzymatic function of affected protein to inhibit growth of bacteria [17-20]. However, the interaction between flavonoids of *Marchantia* species and enzymes still needs in-depth study. Bactericidal effect of flavonoids from *M. linearis* in the present study is comparable to the methanolic extract of *Plagiochila beddomei*, a bryophyte [21]. Similarly, Mukhopadhyay *et al.* [22] evaluated the efficacy of solvent extracts of Himalayan mosses against various bacterial pathogens. Similar findings were also described by Olofin*et al.* [23] related with phytochemicals from the thallus of seven mosses and three liverworts against various pathogens. Sharma *et al.* [24] noticed phenolics as microbicidal against *Staphylococcus aureus* by inhibiting the respiratory enzymes.

SEM images of untreated bacterial cells revealed a surface with minimum wrinkled protrusions. In the present study, the cell membranes of *E. coli* and *S. aureus* are severely affected by flavonoid treatments rather than its cellular content. The penetration of flavonoids in the cell envelope might have caused internal damage to the cells. El-Tarras*et al.* [25] reported that the disintegration of the cytoplasmic membrane by polyphenols in *Escherichia coli* and *Aeromonas hydrophila* leading to excessive loss of viability. The mechanism of action of flavonoids is thought to be degradation of the cell wall, damage to cytoplasmic membrane proteins, the binding proteins, leakage of cell contents, coagulation of cytoplasm and depletion of the proton motive force [26]. There are many possible explanations for these observations. The literature suggests that the active components of the plant extracts might bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and also membrane-bound enzymes. The effects might include the inhibition of proton motive force, inhibition of the respiratory chain components and electron transfer and inhibition of substrate oxidation. Similarly, the other possible impacts are uncoupling of oxidative phosphorylation, inhibition of active transport, loss of metabolites and disruption of synthesis of DNA, RNA, protein, lipid and polysaccharides [25].

The cytoplasmic membranes of bacteria provide barrier to the passage of molecules into and out of the cells. This permeability barrier role of cell membranes is integrated to many cellular functions, including the maintenance of the energy status of the cell; other membrane coupled energy - transfer processes, solute transport and regulation of metabolism and control of turgor pressure. Marked leakage of cytoplasmic materials such as sugar, protein and nucleic acid is considered indicative of gross and irreversible damage of the cytoplasmic membrane. Many antimicrobial compounds that act on the bacterial cytoplasmic membrane induce the loss of 260 nm-absorbing materials (nucleic acid) and 280 nm absorbing material (protein). The bacterial suspension treated with flavonoids of *M. linearis* reveals significant loss of 260 and 280 nm-absorbing materials, suggesting that nucleic acids and proteins are lost through the damaged cytoplasmic membrane. Toxic effects on membrane structure and function have been generally used to explain the antimicrobial action of polyphenols. This resulted in membrane expansion, increased membrane fluidity and inhibition of a membrane-embedded enzyme [27]. Devi and Kapila, [28] reported the antibacterial mechanism as disruption of plasma membrane by the phytochemicals in the extracts of Indian liverworts.

CONCLUSION

Antibacterial potentiality of flavonoid extract was found to be at par with synthetic antibiotics. Gram-positive and Gram-negative bacteria tested showed varying level of minimum inhibitory concentrations.

Scanning and transmission electron microscopy revealed remarkable morphological deformities. The mean of survivors obtained at different concentration plated for each organism showed a decline. Further studies are warranted to isolate and purify the lead molecule and analyze the molecular mechanism of bactericidal potentiality.

Conflict of Interest: The authors declare no potential conflicts of interest exist.

Table 1:Bactericidal activity of flavonoid extracts from *M. linearis*in terms of MIC and MBC against selected bacteria

Pathogens	Flavonoid extract		Ampicilin	
	MIC (mg/ mL)	MBC (mg/ mL)	MIC (mg/ mL)	MBC (mg/ mL)
Staphylococcus aureus (+)	0.06	0.18	0.12	0.25
Bacillus subtilis (+)	0.12	0.36	0.12	0.43
Klebsiella pneumoniae (-)	0.25	0.62	0.25	0.5
Escherichia coli (-)	0.12	0.25	0.12	0.43
Proteus vulgaris (-)	0.5	0.75	0.43	0.5
Salmonella typhimurium (-)	0.62	1.0	0.43	0.75
Bacillus cereus (+)	0.06	0.36	0.12	0.43
Pseudomonas aeruginosa (-)	0.5	1	0.25	0.5
F ratio I ID	6678.67** 20.182**	5965.66** 12.284**	9698.59** 32.342**	8176.29** 17.600**
$CD_{(0.05)}$ I ID	0.057 1.225	0.009 1.501	0.007 1.609	0.005 1.205

*Gram-positive (+) and Gram-negative (-) bacteria. MIC, Minimal inhibitory concentration which corresponds to the minimum *M. linearis* flavonoid concentration capable to inhibit the visible growth of the bacteria. MBC, Minimal bactericidal concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums.

Table 2: *In vitro* time-kill assessment of the flavonoids of *M. linearis* incubated over 8 h

	log_{10} kill (1/2) × MIC			log_{10} kill 1× MIC			log_{10} kill 2× MIC			F ratio I ID	$CD_{(0.05)}$ ID
	0h	4h	8h	0h	4h	8h	0h	4h	8h		
S. aureus	2.187	2.895	3.233	2.207	1.135	0.183	2.244	−0.264	−2.213	798.67** 11.833**	0.416 1.212
B. cereus	2.387	3.512	4.674	2.351	1.391	0.902	2.391	0.681	−1.839	859.85** 12.9556**	0.65 1.291
B. subtilis	2.27	3.392	4.268	2.254	1.109	0.045	2.297	−0.161	−3.968	969.89** 17.838**	0.654 1.361
K. pneumoniae	2.443	3.447	4.142	2.222	1.079	0.652	2.156	0.562	−1.313	798.56** 20.825**	0.252 1.881
E. coli	2.457	3.567	4.012	2.167	0.789	0.567	2.023	0.344	−1.456	699.58** 12.005**	0.298 1.085

Table 3a: Membrane leakage of bacteria in terms of reducing sugar (µg/mg) treated with different concentrations of *M. linearis*flavonoids (50-500 µg/mL)

	E. coli			***S. aureus***			***B. subtilis***			***K. pneumoniae***		
	0	**12**	**24**	**0**	**12**	**24**	**0**	**12**	**24**	**0**	**12**	**24**
Control	0.10	0.10	0.12	0.13	0.14	0.14	0.12	0.12	0.14	0.10	0.14	0.14
50 µg/ mL	0.8	2	4	1	2.8	5	1	3	5.8	1.2	2.9	4.6
100µg/ mL	0.8	2.6	6.7	1.6	3.9	7.6	1.7	5.2	8.5	2	6.2	7.7
200 µg/ mL	1	5.8	9.6	1.7	4.3	8.9	2	7.8	10	2.4	8.2	10
400µg/ mL	1	7.7	11.5	2	6.8	13.5	2.5	10	14	2.6	10	12.2
500µg/ mL	1.9	9.8	14	2.5	10	18	2.8	13.6	17	2.9	13	16
F ratio I		119.65**			82.54**			82.55**			114.54**	
ID		28.66**			17.53**			3.68**			18.79**	
$CD_{(0.05)}$ I		0.096			0.103			0.066			0.066	
ID		0.32			0.72			0.12			0.65	

Table 3b: Membrane leakage of bacteria in terms of reducing sugar (µg/mg) treated with different concentrations of *M. linearis*flavonoids (50-500 µg/mL)

	B. cereus			***P. vulgaris***			***P. aeruginosa***			***S. typhimurium***		
	0	**12**	**24**	**0**	**12**	**24**	**0**	**12**	**24**	**0**	**12**	**24**
Control	0.12	0.13	0.13	0.1	0.11	0.11	0.1	0.12	0.14	0.11	0.12	0.12
50 µg/ mL	1.2	4.6	6	0.8	2	2.8	0.9	2.4	3	0.9	1.2	2.7
100µg/ mL	1.8	6.9	8.5	1.4	3.8	5	2.2	3	4.2	1.6	2.8	3.8
200 µg/ mL	2	8.7	11	1.8	4.6	6.2	3	4.8	6	2.6	4	5.7
400µg/ mL	2.5	9.8	13	2	6	7.2	3.2	5.7	7.8	2.7	6.2	7.2
500µg/ mL	2.7	12.6	17	2.4	6.6	8.2	3.6	7	9.1	2.8	7.3	8.2
F ratio I		129.25**			90.25**			82.4**			94.52**	
ID		7.022**			13.65**			10.33**			12.889**	
$CD_{(0.05)}$I		0.029			0.028			0.09			0.025	
ID		1.271			1.209			1.307			1.403	

Table 4: Effect of *M. linearis* flavonoid extract on respiratory chain dehydrogenase in selected bacterial cells

	Conc. µg/mL	0	15	30	45	60	F ratio I ID	$CD_{(0.05)}$ I ID
S. typhimurium	C	0.9	1.48	2.8	3.6	4.57	94.65** 12.29**	0.128 1.296
	10	0.8	1.56	2.52	2.4	2.4		
	20	0.6	1.8	2.4	2.3	2.1		
	30	0.5	1.6	2.2	2	1.8		
	40	0.5	1.2	2	1.6	1.5		
	50	0.5	1	1.6	1.3	1.2		
S. aureus	C	1	1.6	2.4	2.7	3.6	112.35** 10.05**	0.103 1.011
	10	0.5	1.5	2.2	1.93	1.44		
	20	0.5	1.3	2.4	1.7	0.89		
	30	0.5	1.1	2.2	1.5	0.67		
	40	0.5	0.9	2	1.23	0.6		
	50	0.5	0.78	1.6	1	0.54		
K. pneumoniae	C	0.97	1.08	2.09	3.79	4.09	185.67** 13.36**	0.135 1.092
	10	0.9	1.5	3.4	2.6	2		
	20	0.7	2.4	3.7	2.4	1.7		
	30	0.6	1.9	2.3	2.0	1.35		
	40	0.5	1	1.9	1.48	1.2		
	50	0.5	0.9	1.4	1.2	0.82		
E. coli	C	1.0	1.8	2.9	3.4	4.2	173.53** 18.28**	0.129 1.085
	10	0.5	1.6	2.7	3.2	2.86		
	20	0.5	1.5	2.3	1.7	1.41		
	30	0.5	1.0	1.9	1.57	1.30		
	40	0.5	0.9	1.8	1.3	0.96		
	50	0.5	0.76	1.0	0.96	0.56		
B. cereus	C	0.9	1.08	2.09	3.79	3.99	201.29** 11.12**	0.205 1.072
	10	0.6	1.69	2.94	2.5	1.9		
	20	0.5	1.55	2.45	1.7	1.3		
	30	0.5	1.24	2.2	1.0	0.68		
	40	0.5	1.0	1.4	0.74	0.51		
	50	0.5	0.9	1.3	0.82	0.41		
B. subtilis	C	1.0	1.08	2.09	3.09	3.88	469.28** 12.520**	0.114 1.031
	10	0.6	1.3	2.68	3.62	2.55		
	20	0.5	1.2	2.3	2.9	2.0		
	30	0.5	1.0	2.1	2.4	1.6		
	40	0.5	0.9	1.45	1.0	0.66		
	50	0.5	0.82	1.0	0.76	0.51		
P. vulgaris	C	1	2.08	3.09	3.5	4	355.29** 17.02**	0.098 1.003
	10	0.8	1.4	2.8	3.9	2.76		
	20	0.7	1.4	2.7	3.7	2.25		
	30	0.6	1.3	2.5	3.2	1.62		
	40	0.5	1	1.96	2.1	1.45		
	50	0.5	0.92	1.2	1.08	0.98		
P. aeruginosa	C	1.0	1.5	2.67	3.45	3.99	299.68** 18.25**	0.103 1.007
	10	0.9	1.6	2.5	3.6	2.78		
	20	0.7	1.46	2.7	3.8	2.5		
	30	0.6	1.28	2.4	3.0	1.8		
	40	0.5	1.1	2.0	2.72	1.2		
	50	0.5	0.96	1.52	1.24	1		

Table 5: Determination of leakage of UV_{260} and UV_{280} absorbing material from ***tested bacteria with M. linearis flavonoid extract*** spectrophotometrically

Pathogen		Time in minutes									F ratio I ID	$CD_{(0.05)}$ I ID
		0	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**		
S. typhimurium	UV_{260}	0	0	0	0.03	0.04	0.06	0.08	0.09	0.1	35.55** 7.678**	0.051 1.896
	UV_{280}	0	0.04	0.09	0.1	0.13	0.14	0.15	0.16	0.18	41.66** 2.508**	0.03 1.72
S. aureus	UV_{260}	0	0.08	0.1	0.1	0.1	0.2	0.34	0.38	0.4	29.35** 1.508**	0.708 1.12
	UV_{280}	0	0.2	0.24	0.29	0.3	0.32	0.32	0.32	0.32	47.66** 3.920**	0.02 1.39
K. pneumoniae	UV_{260}	0	0.04	0.07	0.08	0.09	0.12	0.2	0.23	0.3	51.32** 2.828**	0.983 1.29
	UV_{280}	0	0.16	0.2	0.24	0.27	0.29	0.3	0.3	0.3	60.65** 4.209**	0.178 1.97
E. coli	UV_{260}	0	0.03	0.06	0.08	0.1	0.12	0.17	0.22	0.28	38.58** 2.05**	0.222 1.78
	UV_{280}	0	0.13	0.17	0.18	0.19	0.2	0.21	0.24	0.26	60.29** 2.966**	0.552 1.92
B. cereus	UV_{260}	0	0.07	0.09	0.1	0.1	0.15	0.27	0.3	0.35	18.77** 3.960**	0.652 1.12
	UV_{280}	0	0.06	0.08	0.09	0.1	0.16	0.22	0.25	0.28	29.65** 1.465**	0.195 1.38
B. subtilis	UV_{260}	0	0.05	0.08	0.09	0.1	0.13	0.2	0.29	0.34	25.38** 1.269**	0.281 1.11

	UV_{280}	0	0.07	0.09	0.1	0.1	0.15	0.27	0.3	0.35	17.65** 4.88**	0.411 1.98
P. vulgaris	UV_{260}	0	0	0.02	0.04	0.07	0.09	0.1	0.15	0.17	20.30** 2.665**	0.360 1.79
	UV_{280}	0	0.04	0.06	0.09	0.1	0.12	0.14	0.16	0.17	16.69** 4.231**	0.380 1.102
P. aeruginosa	UV_{260}	0	0	0.03	0.04	0.06	0.07	0.09	0.1	0.15	25.32** 2.29**	0.911 1.125
	UV_{280}	0	0.02	0.05	0.07	0.09	0.1	0.12	0.14	0.16	20.68** 2.60**	0.780 1.116

Figure 1. Plant material *Marchantialinearis*Lehm&Lindenb.

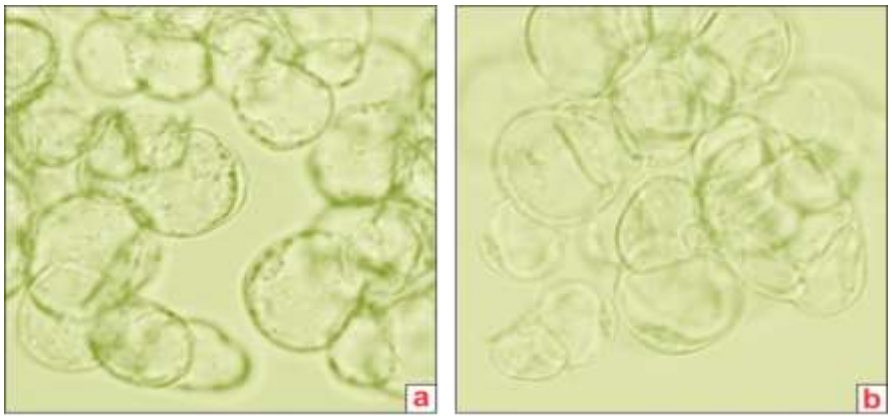

Figure 2a & b: Stereo microscopic photograph of cell aggregates in suspension culture of *M. linearis*

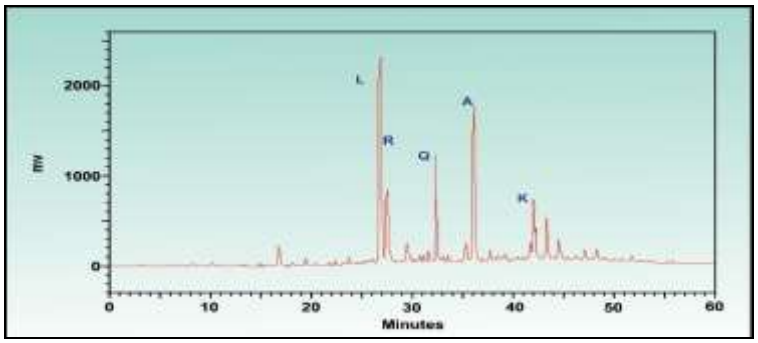

Figure 3: HPLC - PAD Chromatogram of flavonoids of *M. linearis*

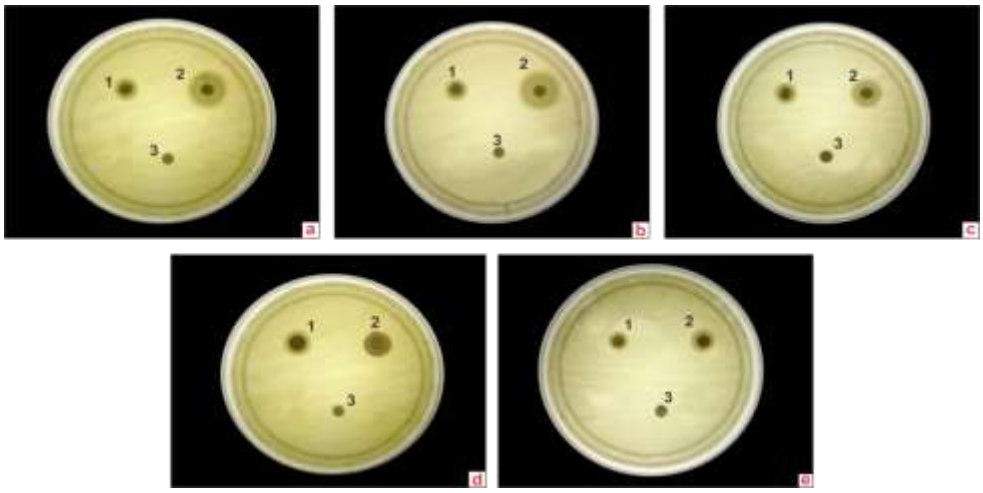

Figure 4a-e: Antibacterial analysis by disc diffusion assay of *M. linearis*a) *Staphylococcus aureus, b) Bacillus cereus, c) Escherichia coli,* d) *Bacillus subtilis* e) *Klebsiella pneumoniae* 1 - 0.125 mg/mL, 2 - 0.25 mg/mL, 3 - control (without extract)

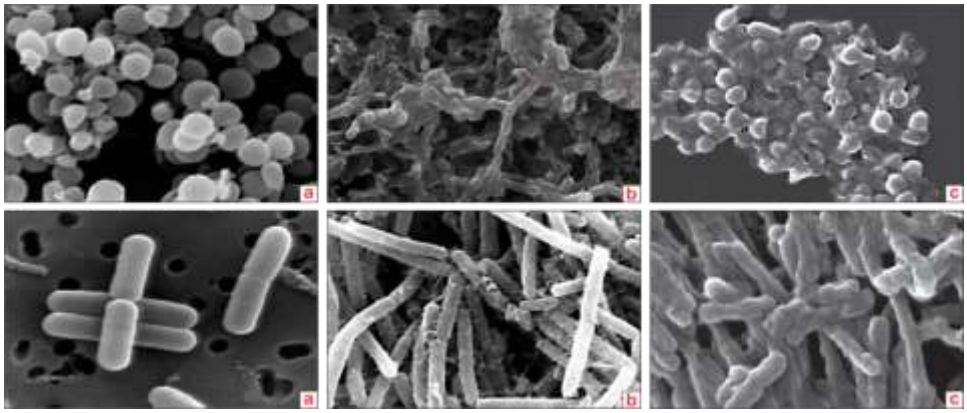

Figure5a-c: SEM analysis of *Staphylococcus aureus* and *Escherichia coli* against flavonoid extract of *M. linearis*a) control, b) 12 h, c) 24 h

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