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Antibacterial potential of Antimicrobial peptides containing whole proteins of two Adiantum species from Himalaya against selected human bacterial pathogens

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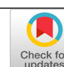
ABSTRACT



Increasing tendency of drug resistance and continuously emerging new diseases have limited the available therapeutic options. Plants are still a positive hope for search of new therapeutic agents, which have provided many effective therapeutic agents and have not been yet explored to their full potential. In the present study, two ferns *Adiantum edgeworthii* and *Adiantum capilllus-veneris* from the Himalayas, have been explored for the presence of antimicrobial peptides (AMPs) in them. Antimicrobial peptides are different class of therapeutic agents having multimodal action mechanism and different sites of action. Hence they could be a solution against the problem of drug resistance. In the present study, whole proteins (WPs) of the ferns were precipitated using TCA, and low molecular weight protein fractions (LMPFs) of WPs were isolated using SDS-PAGE. Antibacterial activities of WPs and LMPFs were tested against six human pathogenic bacteria *S. aureus, S. pneumoniae, E. faecalis*, and *K. pneumoniae, P. aeruginosa* and *E. coli.* The results showed that AMPs were present both the ferns and antibacterial potency of LMPFs was higher than WPs. It was also observed that WPs of *A. capillus-veneris* was about 11.4% more active than WPs *of A. edgeworthii*. Antibacterial activity of WPs was compared with two standard antibiotics (Amoxicillin and Erythromycin) and comparable results were obtained. Further research for purification and characterization of pure antimicrobial peptides from these plants are expected to provide new therapeutic agents.

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INTRODUCTION

Human race is susceptible to various microbial disease, many fatal viral disease like severe acute respiratory syndrome (SARS), Dengue, Ebola emerged in last two-three decades and recently corona virus (COVID-19) infection has taken the entire world in it's grip (Chen *et al.*, 2020). In addition, severity of bacterial diseases has also increase due to development of drug resistance in human pathogenic bacteria, even generally harmless infection of *Staphylococcus aureus* are becoming lethal due to emergence of drug resistant *S. aureus* strains. MDR/XDR tuberculosis is another example of same. Graveness of drug resistance and emergence of new disease become graver due to very slow rate of discovery of new drugs with different mode of action (Spellberg *et al.*, 2004). Discovery of new drug is time and resource intensive, moreover the new drug must be either multimodal in action or must have different

mode/target of action, otherwise microbes would also develop resistant against it. Antimicrobial peptides (AMPs) are the class of therapeutic molecules which are multimodal in action, hence there are very less chance of drug resistance development against them (Gordon *et al.*, 2005).

Nature has always remained source of therapeutics to mankind, since the inception of human civilization. There are different classes of therapeutics majority of which come from Plants, while bacteria and animals contribute comparatively less. Though a large number of therapeutic molecules have been obtained from nature, complete potential of natural sources has not been explored yet. The Himalayas harbor a rich floral biodiversity including prokaryotic forms (cyanobacteria), avascular plant forms (algae, fungi, bryophytes) to vascular plants (Pteridophytes, gymnosperms and angiosperms) (Sharma *et al.*, 2014). Due to cold environment and shade loving nature different Pteridophytes thrive luxuriantly in the Himalaya. Smith, Bold, and Zimmerman divided Pteridophytes into four divisions: Psilophyta, Lycophyta, Sphenophyta and Pterophyta (Axelrod, 1959). Psilophyta are primitive, rhizoidal, aphyllous, homosporous fossils Pteridophytes with dichotomoustlybranced stems, represented by fossils genera only. Lycophyta are microphyllous leave and strobili (cone) bearing, heterosporous and homosporous Pteridophytes, represented by Selaginella and Lycopodium. Sphenophyta are scaly leave bearing, homosporous Pteridophytes, represented by all fossils genera except Equisetum sp. Pterophyta are macrophyllous leave (fronds) bearing, homo and heterosporous Pteridophytes with rhizomatous stem, and commonly called as ferns.

Antimicrobial peptides (AMPs) are naturally produced in response of foreign microbial infections by almost all class of life forms, including prokaryotes to human beings (Gordon *et al.*, 2005). Selectivity, multimodal and multifunctional mode of action, and low propensity of developing resistance against them by microbes make AMPs a suitable therapeutic candidate. Besides, they also show healing and immuno-modulatory activities. AMPs have been isolated from both, animals and plants (Gordon *et al.*, 2005). Ferns of Himalayas can be explored as a source of new AMPs because they are usually not infected by microbial pathogen. Many species of ferns are found globally and none of them has been explored for presence of AMPs in them. Although there are some reports on antimicrobial activities of organic solvents based extracts of ferns but none of them focused on AMPs from ferns. *Adiantum* is a genus of family Pterdiaceae, consisting of almost 250 species. Many ferns of this genus are used in traditional medicinal system worldwide. *Adiantum edgeworthii* and *Adiantum capillus-veneris* (known as Maiden hair fern) are the two species used in present studies for presence of AMPs in them. Six human pathogenic bacteria (three Gram positive: *S. pneumoniae* (MTCC 0655), *S. auerus* (MTCC 1144), *E. faecalis* and three Gram negative *P. aeruginosa* (MTCC 2474), *K. pneumoniae* (MTCC 4040) and *E. coli* were used for checking efficacy of AMPs isolated from these two plants in present study. This is the very first report on antibacterial activities of AMPs from the two ferns *i.e. A. edgeworthii* and *A. capillus-veneris*. from the Himalayas

MATERIALS AND METHODS

Ferns Collection and identification

Ferns were collected from Chauras (District-Tehri Garhwal) and Srinagar Garhwal (District Pauri Garhwal) of Uttarakhand (Figures 1 and 2). The plant material was identified by Forest Research Institute, Dehradun, India.

Procurement and maintenance of bacteria

Four standard culture; *Staphylococcus auerus* (MTCC-1144), *Streptococcus pneumoniae* (MTCC-655), *Pseudomonas aeruginosa* (MTCC-2474) and *Klebsiella pneumoniae* (MTCC-4040) were procured from Microbial Type Culture Collection (MTCC) and Institute of Microbial Technology (IMTECH), Chandigarh, India. Two clinical isolates *E. coli* and *Enterococcus faecalis* were collected from V. C. S. G. Government Institute of Medical Science and Research, Srikot-Ganganali, respectively. The cultures were stored at 4 °C as stock cultures and sub-cultured at regular intervals for maintaining their viability.

Inoculums preparation

For antibacterial assays, the stock culture of each bacterium was revived by streaking it on nutrient agar plates, followed by incubation at 37°C. For each bacterium, a well separated colony was picked with the help of inoculums loop and re-suspended in 10 ml normal saline to match the optical density equivalent to 0.5 McFarland standards. The suspensions, thus prepared were used as inoculums for antibacterial activity assays, respectively for each bacterium, using Agar well diffusion method.

Extraction of Whole proteins (WPs) from fern fronds

Plant leaves (Fronds) were first clean with distilled water in order to remove all dirt and debris. Green parts of fronds were plucked and 1 gm of

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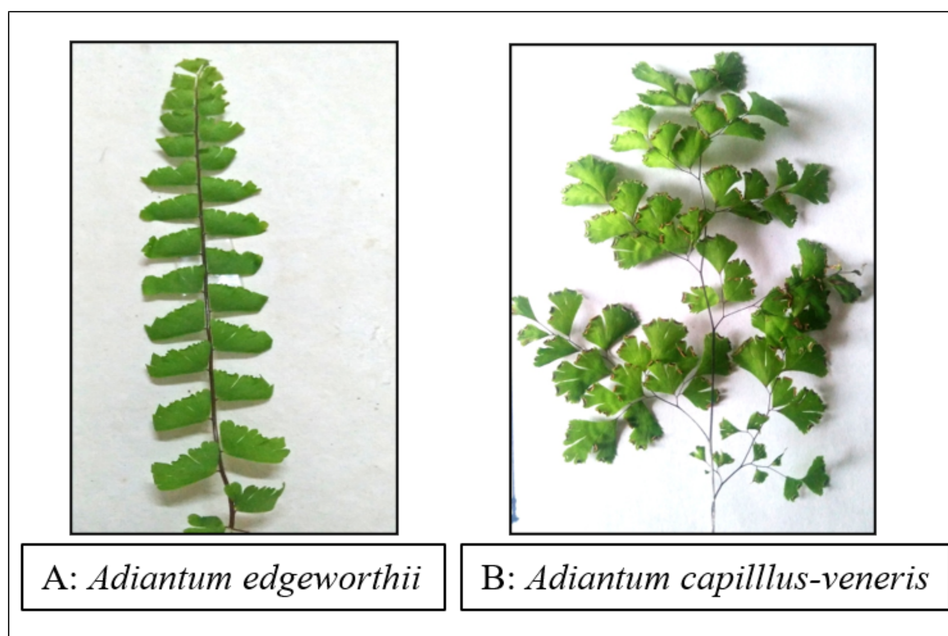

Figure 1: Fronds of (A) Adiantum edgeworthii and (B) Adiantum capillus-veneris

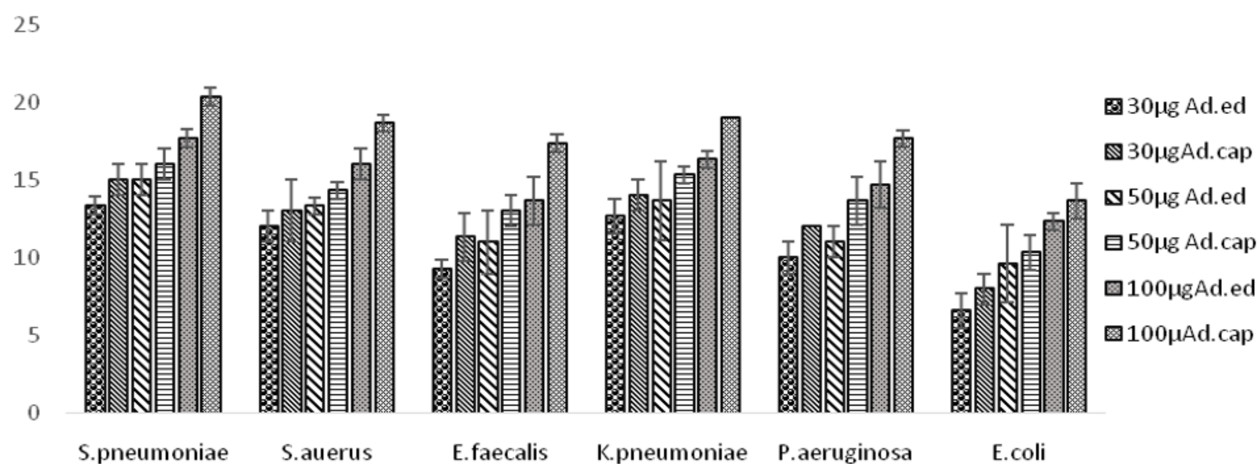

Figure 2: Graph showing antibacterial activities of WPs dissolved in AU of both the ferns against selected bacteria

Table 1: Proteins yield from 1 gm of green fronds of both the ferns, during their different growth stages

Growth stages	*A. edgeworthii*	*A. capillus-veneris*
Fast vegetative growth	4,050 μg	13,800 μg
Reproductive stage	2,900 μg	6,050 μg
Decay and desiccation	402 μg	4430 μg

Table 2: Antibacterial activities of WPs, dissolved in SLB, against selected bacterial pathogens

Bacteria	Zone of inhibition with WPs in SLB (in mm)					
	(*A. edgeworthii*)			(*A. capillus-veneris*)		
	Amount of WPs per well			Amount of WPs per well		
	30μg	50μg	100μg	30μg	50μg	100μg
S. pneumoniae	9.33 ± 1.52	5 ±1.0	12.33 ±2.51	10.33 ±1.52	9.66 ±2.08	10.66 ±1.52
S. auerus	18 ±2.0	20 ±2.64	25 ±3.0	18.66 ±3.21	15 ±3.0	25.33 ±0.57
E. faecalis	2.66 ±1.52	4.66 ±2.51	6.66 ±2.88	3.66 ±2.08	4 ±1.0	6 ±2.0
K. pneumoniae	6 ± 1.0	4 ±1.0	10.66 ±2.08	6.66 ±0.57	4.33 ±1.52	9.66 ±1.15
P. aeruginosa	12.66 ±2.08	12.66 ±2.08	15.66 ±1.52	12.66 ±2.08	14.66 ±1.15	15.33 ±1.52
E. coli	4.33 ±1.52	5.33 ±2.08	8.66 ±2.30	5 ±2.0	8 ±2.0	8.33 ±1.52

Table 3: Antibacterial activities of WPs, dissolved in AU, against selected bacterial pathogens

Bacteria	Zone of inhibition with WPs in AU (in mm)					
	(*A. edgeworthii*)			(*A. capillus-veneris*)		
	Amount of WPs per well			Amount of WPs per well		
	30μg	50μg	100μg	30μg	50μg	100μg
S. pneumoniae	13.33 ±0.57	15.0 ±1.0	17.66 ±0.57	15.0 ±1.0	16.0 ±1	20.33 ±0.57
S. auerus	12.0 ±1.0	13.33±0.57	16.0 ±1.0	13.0 ±2.0	14.33 ±0.57	18.66 ±0.57
E. faecalis	9.33 ±0.57	11.0 ±2.0	13.66 ±1.52	11.33 ±1.52	13.0 ±1.0	17.33 ±0.57
K. pneumoniae	12.66 ±1.15	13.66 ±2.51	16.33 ±0.57	14.0 ±1.0	15.33 ±0.57	19.0 ±0
P. aeruginosa	10.0 ±1.0	11.0 ±1.0	14.66 ±1.52	12.0 ± 0	13.66 ±1.52	17.66 ±0.57
E. coli	6.66 ±1.15	9.66 ±2.51	12.33 ±0.57	8.0 ±1.0	10.33 ±1.15	13.66 ±1.15

Table 4: Anti-bacterial activities of AMPs rich LMPFs from *A.edgeworthii* and *A. capillus-veneris*, against selected bacterial pathogens

Bacteria	Zone of inhibition with AMPs rich LMPFs (in mm)			
	A. edgeworthii		*A. capillus-veneris*	
	LMPFs (14μg/well)	Whole proteins ((30μg/well)	LMPFs (10μg/well)	Whole proteins (30μg/well)
S. pneumoniae	8.00±1.15	13.33 ±0.57	13.0±0.57	15.0 ±1.0
S. aureus	6.0±1.50	12.0 ±1.0	11.00 ±0.57	13.0 ±2.0
E. faecalis	0	9.33 ±0.57	0	11.33 ±1.52
K. pneumoniae	9.00±1.50	12.66 ±1.15	14.0±0.57	14.0 ±1.0
P. aeruginosa	19.00 ±2.00	10.0 ±1.0	21.00 ±1.50	12.0 ± 0
E. coli	0	6.66 ±1.15	0	8.0 ±1.0

Table 5: Antibacterial activity of Amoxicillin against selected bacterial pathogens

Bacteria	Zone on inhibition (in mm)			
	*Amoxicillin (antibiotic disc) (30μg/disc)	$Amoxicillin (antibiotic tablet) (100μg/well)	*A. edgeworthii* (30μg/well)	*A. capillus-veneris* (30μg/well)
S. pneumoniae	#	13.0± 0.57	13.33 ±0.57	15.0 ±1.0
S. aureus	28-36	17.0 ± 0	12.0 ±1.0	13.0 ±2.0
E. faecalis	#	11.0 ± 0	9.33 ±0.57	11.33 ±1.52
K. pneumoniae	#	15.0 ± 1	12.66 ±1.15	14.0 ±1.0
P. aeruginosa	#	15.0 ±0.57	10.0 ±1.0	12.0 ± 0
E. coli	17-22	15.0 ±1.15	6.66 ±1.15	8.0 ±1.0

Value not given, *using antibiotic disc from Himedia, $ using commercial antibiotic tablet

Table 6: Antibacterial activity of Erythromycin against selected bacterial pathogens

Bacteria	Zone on inhibition (in mm)			
	*Erythromycin (antibiotic disc) (15μg/disc)	$Erythromycin (antibiotic tablet) (10μg/well)	*A. edgeworthii* (30μg/well)	*A. capillus-veneris* (30μg/well)
S. pneumoniae	26-32	31.0 ±1.15	13.33 ±0.57	15.0 ±1.0
S. aureus	22-23	19.0 ± 2.15	12.0 ±1.0	13.0 ±2.0
E. faecalis	#	17.0± 0.57	9.33 ±0.57	11.33 ±1.52
K. pneumoniae	#	22.0 ± 1.5	12.66 ±1.15	14.0 ±1.0
P. aeruginosa	#	20.0 ± 0.57	10.0 ±1.0	12.0 ± 0
E. coli	#	21.0 ± 1	6.66 ±1.15	8.0 ±1.0

#Value not given, * using antibiotic disc from Himedia, $ using commercial antibiotic tablet

green frond was grinded into fine powder using liquid nitrogen, with the help of pre-chilled mortar-pestle. Trichloroacetic acid (TCA)-Acetone method was used for extraction of total proteins (Isaacson *et al.*, 2006) with slight modification. Briefly, fine grinded fronds were suspended into extraction buffer (0.015M Tris,0.25M EDTA , 0.5M Thiourea and 0.5ml β- mercaptoethanol, pH 7.2) for protein extraction. Suspension was centrifuges at 6000rpm for 15 min to pellet insoluble debris and soluble supernatant was collected in a separate tube. Proteins were precipitated from clear supernatant using 10% (v/v) of 100% Trichloroacetic Acid solution, after gradual addition of TCA followed by overnight incubation at 4°C. Next day, the precipitate was centrifuged at 15,000 rpm for 20min at 4°C. Supernatant was discarded and pellet was washed with chilled acetone for three times by resuspension and centrifugation, air dried and stored at 0°C for future use.

Protein fractionation according to their molecular weight range

WPs were fractioned in four different molecular weight range fractions using SDS-PAGE. WPs of each fern were fractionated by loading 40μg of it in all 10 wells of a SDS-PAGE gel. 12% gel mini gel was

casted and run at 110 volt constant voltage. Electrophoresis was stopped before the dye front reach at the lower end of the gel. Gel was removed from gel caste and quick washed with distilled water for 3 times. Gel was cut horizontally into four parts and protein from each cut parts was eluted and used as separate protein fractions.

Elution of fractionated Proteins (FPs)

For protein elution, gel strips were rinsed with 0.02 M PBS (pH 7.2) for 10 min followed by rinsing the gel with 10 mM EDTA. Gel was further rinsed with 0.1% Triton X-100 to remove excess SDS and proteins renaturation. Gel strips were crushed into small pieces, immersed into minimum volumes of elution buffer, so that protein could diffuse into elution buffer from gel. Gel pieces containing tubes were shaked for 5 minutes. After shaking, proteins in elution buffer were recovered using micropipette and process was repeated twice. Elution buffer from all three times, containing eluted proteins in it, was pooled, lyophilized and stored at 0°C for further uses. Antibacterial activities of protein fractions were also tested as given in section 2.6.1.

Protein estimation of whole proteins (WPs)

The protein content of WPs was measured by the Modified Lowry's Method. Bovine serum albumin (BSA) solution of 1mg/ml concentration was used as the standard. Absorbance was measured at 750 nm in UV-VIS spectrophotometer.

Protein estimation of Low molecular weight Protein fractions (LMPFs)

Concentrations of LMPFs, eluted from cut gel strips were measured by taking absorbance of eluted protein samples at 280 nm, before lyophilization. Due to smaller amount of proteins, concentration of proteins fractions was not measured using Modified Lowry's methods.

Protein extracts (WPs) solubilization in different solubilization mediums

For antibacterial activity testing, WPs was dissolved in either of sample solubilization medium: (i) SDS-lysis buffer (SLB) [0.5 M Tris, 0.33% SDS and 16.6% (v/v) Glycerol] or (ii) Aqueous solution of 125 mM urea. 30μg, 50μg and 100μg of proteins, dissolved in equal volumes of 100μl each was poured into the wells, cut into MHA agar plats.

Comparison of antibiotic activities of WPs with standard antibiotics

Two standard antibiotics, Amoxicillin and Erythromycin were used to compare the antibacterial potential of WPs with them. Amoxicillin was dissolved in dissolved in 1:1 (v/v) solution of PBS (phosphate saline buffer) and 0.1M Sodium Hydroxide. Erythromycin (10 mg/ml) dissolved in Di-Methyl Sulfonium Oxide (DMSO). Initially stocks solutions of 5mg/ml for both the antibiotics were prepared, which was diluted according to experimental requirements. Blank solutions without antibiotics (DMSO for Erythromycin and, PBS with NaOH for Amoxicillin) were used as negative control.

Agar well diffusion method

The antibacterial activity of the WPs was determined by Agar well diffusion. 3.8 gm of Mueller Hinton agar (MHA) media was dissolved in 100 ml of distilled water and autoclaved. Autoclaved media was transferred into sterile petriplates and allowed to solidify. Antibacterial activity of each bacteria was tested by spreading 50 μl of the inoculums (prepared as section 2.5) on separate MHA (Hi-media) plates using sterile swab. For antibacterial activity, 6.0mm diameter wells were cut onto MHA agar plates, already inoculated with bacteria. Plates were incubated at 37°C for appearance of zone of inhibition around the wells. The wells having antimicrobial agents (WPs, LMPs or antibiotics) inhibit the microbial growth and the clear zones were formed around wells. The zone of inhibition was measured in millimeters, with the help of scale having least count 1.0 mm. Assays were performed in triplicates and zone of inhibition were calculated by subtracting the diameter of well from the diameter of clear zone around well.

Broth dilution method for MIC

Serial dilution method was used to get an estimate of Minimum inhibitory concentration (MIC) of WPs against the tested microbial pathogen. In this method 3.0 ml of sterile nutrient broth media was taken into test tubes and graded doses of WPs, dissolved in equal volume of 100μl were added. Then these test tubes were inoculated with the bacteria. Suspension of tested bacterium having concentration of 5x10^5cfu/ml, was prepared in nutrient broth containing tubes. This was achieved by ten-fold dilution of bacterial suspension having O.D. equivalent to 0.5Mc Farland standard and adding 50 μl of this suspension to 3.0 ml of nutrient broths. 10 μg/ml,20 μg/ml,40μg/ml,50 μg/ml, 100 μg/ml, 200 μg/ml ,500 μg/ml to 1mg/ml concentration of WPs were used for MIC testing and tubes were incubated at 37°C for 24 hours.

RESULTS AND DISCUSSION

Plant identification and relevance for their selection

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Two ferns used in present study were identified as *Adiantum edgeworthii* (Figure 1. A) and *Adiantum capillus-veneris* (Figure 1. B) from genus *Adiantum*. In present study these two ferns were analyzed for presence of AMPs in them. Ferns, a class of Pteridophytes, have not been explored for presence of AMPs in them and are normally not affected by microbial pathogens hence could provide a different class of AMPs. Ethanobotanical and Ethno medicinal importance of these plants have been investigated and studied by various authors. *Adiantum* has been reported for its various medicinal properties, specially antibacterial and antiviral, antifungal activities. Antibacterial activity of organic solvent extracts of different *Adiantum* species against *P. aeruginosa* (Thomas, 2017; Zhang *et al.*, 2019), *E. coli* (Johnson *et al.*, 2017) , *K. pneumonia*e (Johnson *et al.*, 2017; Reshi *et al.*, 2017), and *S. aureus* (Reshi *et al.*, 2017; Thomas, 2017), *Streptococcus agalactia* (Reshi *et al.*, 2017), *Serratia marcescens* (Thomas, 2017), *Micrococcus lysodeikticus* (Zhang *et al.*, 2019), *Bacterium paratyphosum* B (Zhang *et al.*, 2019), *Bacillus subtilis* have also been reported by various research groups.

Protein yield and seasonal variations.

Whole proteins (WPs) were extracted at different growth stages of both the ferns; fast vegetative growth stage, reproductive (spore forming) stage and desiccation stages. Reproductive stage is differentiated from vegetative growth stage by presence of sporangia on margins of fronds (Figure 1. B), and desiccation stage is identified by drying of green parts of plants. Protein yield from all three stages are shown in Table 1. Highest protein yield was observed during profuse growth stages of plant which was almost double from the spore forming stages, while least protein was obtained during decayed stage of ferns. Protein yield during reproductive season dropped to almost half of the vegetative growth season, because during reproductive phase vegetative growth ceases and sporogenesis starts. During vegetative growth, protein synthesis is at maximum rate to support the newly forming cells and cell organelles. Hence, highest protein content was observed during vegetative growth stage. During reproductive stages, vegetative growth is dawdling and metabolic activities are diverted for spore formation, hence protein yield at this stage was lower at this stage compared to vegetative stage but higher than decaying stage. During decay stages cytoplasm of cells start shrinking, new cell formation and metabolic activities are ceased. Although both the ferns belong to same genus *Adiantum* and grows under similar conditions but protein content of *A. capillus-veneris* was higher than *A. edgeworthii* for same amount of green front tissue. *A. capillus-veneris* shows more luxuriant growth and green fronds compared to *A. edgeworthii*, hence more proteins content than *A. edgeworthii*, to support higher leafy growth.

Solubilizaiton of WPs in different medium and their effect on antibacterial activity

For antimicrobial activity testing by disc-diffusion method, WPs were dissolved in two types of protein solubilization medium, SLB and AU. Initially WPs were dissolved in SLB, but the result of agar well diffusion based antibacterial activities were neither consistent nor dose dependent. Hence, WPs were dissolved in aqueous solution of urea (AU), which is a good chaotropic agent. In the literature use of urea (9 molar concentrations) or urea with thiourea (7 molar urea, 2 molar thiourea) containing buffer are recommended for better protein solubilization. In present study, 125mM concentration of urea was used instead. This was done to avoid any false positive results caused by used of 9 M urea or 7M urea with 2M thiourea. 125 mM solution of urea in water served the solubilization purpose nicely, hence further high concentrations of urea were not used. (Akeel *et al.*, 2018) also compared two different solubilization buffers for extraction of antimicrobial proteins from seeds of *Cucumis sativus L.* Although they did not study the effect of different buffers on solubilization of proteins for antimicroassay, but the requirement of better protein solubilization buffer was the reason for using two different buffers by (Akeel *et al.*, 2018). Results of antimicrobial activity after solubilization of proteins in SLB and AU are shown in Tables 2 and 3, respectively. As expected the results in Table 3 (WPs dissolved in AU) were consistent and dose dependent.

Antibacterial activity of whole proteins

Antibacterial activities of WPs were measured using Agar well diffusion assay, appearance of zones of inhibition around the agar wells, indicated presence of antibacterial activity. As mentioned above for agar well diffusion assay WPs were dissolved in two types of medium, SLB and AU, results of which are given in Tables 2 and 3.

For WPs of both *A. edgeworthii* and *A. capillus-veneris*, maximum and minimum zones of inhibition (ZOIs) were observed against *S. pneumoniae* and *E. coli*, respectively. Overall order of antibacterial activity (diameter of ZOIs) against all the six bacteria was observed as: *S. pneumonia* > *K. pneumonia* > *S. aureus* > *P. aeruginosa* > *E. faecalis* > *E. coli* using WPs of both the ferns as antibacterial agents, separately. Other research groups also studies the effect of antimicrobial proteins and pep-

tides extracted from other plants sources on human pathogenic bacteria, including the three; *S. aureus, P. aeruginosa* and *E. coli*. Like our observations, crude proteins isolated from *Allium cepa* (Garlic) and *Peltophorum dubium* were also more effective against *S. aureus* and *P. aeruginosa* compared to *E. coli* (Gao *et al.*, 2019; Rodríguez-Decuadro *et al.*, 2018). Even for peptides being expressed in gene expression system, the crude proteins were more effective against *S.aureus* (25% growth reduction) than E. coli (20% growth reduction) as bactericidal agent (Chahardoli *et al.*, 2018; Mandal *et al.*, 2009) also reported similar pattern of antibiotic potential against *S. aureus, P. aeruginosa* and *E. coli*, using partially purified antimicrobial peptides peak from Green coconut water proteins extract. They reported that MIC of most two reverse phase HPLC peaks (F-3 and F-1) against *E. coli* were 302μg/ml and 82 μg/ml, which was higher compared to *S. aureus* (274 μg/ml and 80 μg/ml) and *P. aeruginosa* (259 μg/ml and 79 μg/ml). In their experiment, peak F-3 was having maximum protein amount but lowest amount of AMPs in it, and the peak F-1 was having lowest protein amount but highest concentration of AMPs. Antibacterial activity of peak F-3 (less AMPs but more other proteins) was higher against *S. aureus* and *P. aeruginosa* than *E. coli*, the same results were observed in our study, where anti-bacterial activities of WPs was higher against S. *aureus, P. aeruginosa* compared to *E. coli*. (Ruiz-Ruiz *et al.*, 2017) also observed the same pattern with AMPs isolated from fruits of *Bromelia pinguin L*. They observed that activity of crude soluble proteins fraction was more against *S. aureus* (13.0 $\pm$ 0.40) than *E. coli* (12.0 $\pm$ 0.30). A reverse pattern was observed when purified protein fractions of the same were tested, where ZOI against E. coli was 8.0 $\pm$ 0.50mm, while no zone was observed against *S. aureus*. Similar pattern of opposite antimicrobial activities of crude protein extracts and enriched/purified protein fractions was observed in present study and other studies (Mandal *et al.*, 2009). Study by Jabeen and Khanum (2017) showed contradicting results, where *E. coli* was more sensitive than *S. aureus* and *P. aeruginosa*, for crude protein extracts, ammonium sulfate precipitate as well as peptide concentrated fractions of *Momorica charantia*. MIC of the WPs estimated using broth dilution method showed a range of 20μg/ml -900ug/ml against all the bacteria, although MIC of WPs from *A. capillus-veneris* was lesser compared to *A. edgeworthii* confirming higher antibacterial potential of WPs of former.

Graphical representation of antibacterial potentials (indicated by diameter of zone on inhibition) of WPs, of both the fern species (Figure 2) showed that average antibacterial activity of WPs from *A. capillus-veneris* proteins was 11.4% higher than that of *A. edgeworthii*, against all the tested bacteria. This could be due to difference in composition and/or structure of AMPs present in WPs of both the ferns causing difference in antibacterial activities of both the ferns for equal amount of protein. Although, both the ferns belong to same genus and grows under similar conditions but due to difference in species their genetic material would be different, accounting for difference in antimicrobial potential.

Statistical analysis of data showed that for 50μg/well of WPs, there was no significant statistical difference in activities of both the ferns (at 5% significant level; p value calculated for t-test using MS-excel was 0.07, which is higher than 0.05 hence no significant difference). For 30μg and 100μg of WPs the differences between antimicrobial activities of the two ferns were statistically significant (at 5% significant level; p values calculated for *t*-test using MS-excel were 0.037 and 0.0006, which is lesser than 0.05 hence significantly different).

Elution of low molecular weight proteins fractions (LMPFs) and their antibacterial activities

Low molecular weight rich protein fractions (LMPFs) were isolated from WPs of both the ferns using SDS-PAGE. Antimicrobial activities of eluted LMPFs are shown in Table 4. No activity was observed against *E. faecalis* and *E. coli* in LMPFs of both the ferns. Unlike whole proteins, the maximum activity with LMPFs was observed against *P. aeruginosa* while minimum activity was observed against *S. aureus*, for both the *Adiantum* species.

ZOIs values given in Table 4 indicate that eluted LMPFs from both ferns showed better antibacterial activity than their WPs. Half of the concentration of LMPFs (15μg) of *A. edgeworthii* showed comparable effect with that of double the amount (30μg) of WPs. The effect was more significant for *P. aeruginosa* for which half concentration of LMPFs showed almost double ZOI. (Mandal *et al.*, 2009) observed that antibacterial activity of with reverse phase HPLC isolated peaks the peak F-1 was highest than other two peaks F-2 and F-3, while protein concentration was highest in peak-3. Presence of other proteins may mask the activity of AMPs hence crude protein extracts or peaks containing higher amount of proteins are less active compared to proteins fractions or peaks having low protein but higher percentage of AMPs in them. Observations showed that Gram negative bacterial were more sensitive to LMPFs than Gram positive bacteria. (Adinortey *et al.*, 2013) observed the same phenomenon with

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many antibiotics. (Britto *et al.*, 2012; Hazarika and Sood, 2015) observed the same with organic solvent extracts of ferns, where they found that Gram negative bacteria are more susceptible than Gram positive bacteria. They speculated the different cell wall structure of both Gram positive and Gram negative bacteria is responsible for it. This fact also confirmed that AMPs acts upon cell membrane and outer membrane of Gram negative bacteria may be an easy target for AMPs action compared to Gram positive ones.

In case of LMPFs from *A. capillus-veneris*, one third concentration of LMPFs (10μg) produced comparable effect to the three times amount (30μg) of whole proteins of it, and effect was more significant with *P. aeruginosa*. Thus, average activity of LMPFs could be considered approximately 30-40% better than their whole proteins. Elution of proteins from lower side of gel provided a protein fraction which was rich in low molecular weight range proteins. AMPs are also low molecular weight peptides molecules hence the LMPFs were rich in AMPs and showed better activity.

Antibacterial activity comparison of WPs and LMPFs revealed another interesting fact that while the WPs were more effective upon Gram positive bacteria, the LMPFs were more effective against Gram negative ones. Same fact was observed by others (Yu *et al.*, 2016). Purified peptides from *Spirulina platensis*, a cyanobacteria, were found more microbicidal against *E. coli* (MIC 8mg/ml) than *S. aureus* (16mg/ml) (Sun *et al.*, 2016). Although, the findings of (Rodríguez-Decuadro *et al.*, 2018) were opposite to it. They found that same concentration (14.4μg/ml) of purified peptides from *Peltophorum dubium* was inhibiting 56% growth of *S. aureus* but unable to inhibit growth of *E. coli*.

Comparison of antibacterial activities of WPs, Amoxicillin and Erythromycin

Antibacterial activities of WPs were compared with two standard antibiotics, Amoxicillin and Erythromycin, using agar well diffusion assay. Antibiotic tablets were purchased from commercial market; Amoxicillin 500mg (Alkem laboratories Pvt Ltd) and Erythromycin 500mg (Abbot Healthcare Pvt Ltd). Amoxicillin showed maximum zone of inhibition against *S. aureus* and Erythromycin showed maximum ZOI against *S. pneumoniae*. Both the antibiotics showed minimum zone of inhibition against *E. faecalis* for same concentration. Erythromycin was more effective than amoxicillin against all the bacteria. Results of antibacterial activities of both the antibiotics are shown in Tables 5 and 6.

Amoxicillin, exerts is activity by interfering the process of cell wall synthesis of the bacteria. ZOIs with Amoxicillin range from 11.0$\pm$0-17.0$\pm$0 mm, with 100 μg antibiotics per well (Table 4). According to standard antibiotic sensitivity table, released by Hi-Media laboratories (Humphries *et al.*, 2018) 30 μg of Amoxicillin gives 17-22mm ZOIs with *E. coli* and 28-36 mm ZOIs with *S. aureus* (Table 5). These ZOIs are higher than the observed ZOIs during our experiment (17-22mm vs 15mm, and 28-36 mm vs 17mm) despite three times dose of antibiotic was used in our experiment (100μg compared to 30μg) the observed ZOIs were lesser than standard, hence it can be assumed that antibiotic content of the commercial antibiotic tablet may be one third of the tablet weight, or it may be due to its incomplete solubilization in PBS-NaOH buffer too. Comparison of antibiotic activities of whole proteins and amoxicillin shows that antibacterial activities of 30μg of whole proteins (considered equivalent to 100μg of commercial amoxicillin tablet) was almost equivalent the antibacterial activity of Amoxicillin (for *S. pneumoniae*, *E. faecalis* and *K. pneumoniae*). For *S. aureus, P.aeruginosa* and *E. coli*, 100μg of whole proteins showed antibacterial activity almost equivalent to 30μg Amoxicillin from commercial Amoxicillin tablet. Lesser activity of whole proteins of ferns could be accounted for presence of other proteins in it rather than only antimicrobial peptides.

Erythromycin inhibits protein synthesis by binding to the 23S rRNA molecule in the 50S subunit of ribosomes, thus stops the growth of bacteria. ZOIs with Erythromycin range from 17.33$\pm$0.57-31.33$\pm$1.15mm, with 15 μg antibiotics per well (Table 6). According to standard antibiotic sensitivity table, released by Hi-Media laboratories, (Humphries *et al.*, 2018) 15 μg of erythromycin gives 26-32mm ZOIs with *S. pneumoniae* and 22-23 mm ZOIs with *S. aureus* (Table 5). These ZOIs are almost equal to the observed ZOIs during our experiment (26-32 mm vs 31mm, and 22-23 mm vs 19mm. Comparison of antibiotic activities of whole proteins and erythromycin shows that antibacterial activities of 30μg of whole proteins were lesser than even 10μg of commercial antibiotics, for all the bacteria used in present study. This could also be due to presence of other proteins and lesser amount of AMPs in WPs.

Activity of AMPs was very much less compared to Erythromycin; a protein synthesis inhibiting antibiotic, for all six bacteria used in the study. These comparisons clearly indicated that any cell wall synthesis inhibiting agent is present in WPs of both the ferns. Studies by (Battersby *et al.*, 2016; Coutinho *et al.*, 2008) showed that antibacterial proteins con-

tain AMPs in them, which account for their antibacterial activity. Their findings support our concept that antibacterial activities of whole proteins fractions was also due to presence of AMPs in them. In addition comparable activity of whole proteins of ferns and Amoxicillin, a cell wall synthesis inhibiting antibiotic, also confirms presence of AMPs in whole proteins of both the ferns.

CONCLUSIONS

AMPs are different class of antimicrobial agents which are multi-modal in actions. Although, oral delivery of AMPs as therapeutic agents is a big question, because of proteolytic environment of human alimentary canal. Rapidly increasing drug resistance in bacteria against commonly used antibiotics has propelled scientific community to explore newer alternative of antibiotics. Although AMPs are prone to proteolysis and high temperature, their multi-targeted mode of action make them an ideal antimicrobial candidate. Ferns are relatively new to be explored for AMPs, two of the ferns *A. edgeworthii* and *A. capillus-veneris* were explored for presence of AMPs in them. Proteins from both of these ferns showed antibacterial activity against the selected bacteria used in study and experiments confirmed presence of AMPs in them. A distinct changes in antibacterial potential of whole proteins from both the ferns showed that both ferns differs in their AMPs composition. Superior antibacterial activity of low molecular weight protein rich fractions (LMPFs) of whole proteins extracts further confirmed presence of AMPs in them. Due to limitation of resources we could not isolate and find the sequence of AMPs from these ferns and in our further studies this step would be addressed. Further work will accentuate the isolation and characterization of purified AMPs from WPs of different ferns. Although not against normal pathogenic bacteria, but AMPs can be a good answer to drug resistant bacteria, against which the world wide researches are going on but the problem is still persisting and the gravity of problem is increasing continually.

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