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Bioactive compounds from marine red alga *Acanthophora spicifera* associated bacteria against diabetic foot ulcer derived bacterial isolates

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Abstract

Infection by extremely drug resistant pathogens increases the risks associated with diabetic foot ulcers and there is a need for new antibacterial agents to treat them. In this study, biocompounds from heterotrohic bacteria associated with autotrophic red alga *Acanthophora spicifera* were tested for antibacterial and antibiofilming potency against pathogens isolated from diabetic foot ulcers. Pus samples from thirty diabetic foot ulcers from a tertiary care hospital were processed for microbiological examination and antibiogram test. Epiphytic and endophytic heterotrophic bacteria isolated from red alga were screened for antagonistic activity against the extensively drug resistant pathogens using dot plate assay and cross streak assay. Extracts of potential bacteria were tested for anti-pathogenic, anti-biofilming and anti-inflammatory properties. Gas chromatography- mass spectrometry analysis on crude bioactive extract was carried out. Ulcers revealed polymicrobial type infection. The antibiogram study revealed extremely drug resistant extensively drug resistant pathogens in 15% of isolates. Among 28 endo and exo symbiotic heterotrophic bacterial isolates, an epiphytic *Bacillus* was found highly antagonistic to extensively drug resistant pathogens. Bacterial extract proved bactericidal with minimum inhibitory concentration value of 64 µg/mL for Gram positive pathogens and 128 µg/ml for Gram negative pathogens. The extract inhibited biofilming of pathogens at 1000 and 500 ppm levels. Gas chromatography- mass spectrometry analysis of crude extract revealed compounds having antibacterial, antibiofilming and anti-inflammatory activities. The study suggests that *Acanthophora spicifera* associated *Bacillus* may serve a good source of pharmaceutical lead in designing drugs to treat and cure diabetic foot infection due to extensively drug resistant pathogens.

Keywords: Extensively drug resistant pathogens, *Acanthophora spicifera* extracts, Epiphyte, Endophyte, Antipathogenic, Antibiofilming, Anti inflammatory property

1. Introduction

Over the last century, due to changing lifestyle and food habits various chronic diseases like diabetes are causing serious concern to human health. Many diabetic patients have the chance of developing foot ulceration (DFU). DFUs when associated with multi-drug resistant (MDR) or extensively drug resistant (XDR) pathogens make treatment difficult [1].

Bacterial proteases at infection site may disrupt host leukocytes and bring about the release of the lysosomal constituents of neutrophils to cause inflammatory response. Release of lysosomal enzymes causes further tissue damage and subsequent inflammation. The lipoxygenase enzymes in lysosomal contents process the formation of leukotrines and prostaglandins that are inflammatory agents. Lipoxygenase catalyzes deoxygenation of polyunsaturated fatty acids to produce leukotrienes, which are essential mediators in a variety of inflammatory events [2]. Hence, substances that contribute to antimicrobial properties with inhibitory activity against injurious enzymes shall be important in the event of retarding the progression of inflammation.

In the existing demand for the need of novel therapeutics to cure diabetic complications, the attention of research scientists has been focused to marine organisms that have developed unique metabolic abilities to ensure

their survival in diverse and hostile habitats. An array of secondary metabolites with specific activities against human pathogens can be produced by algal associated epiphytic and endophytic microbes [3].

Literature review specifies that *Acanthophora spicifera* is autotrophic marine algal red seaweed with antibacterial and antiviral activity [4]. Ethanol extracts showed good antifungal activity [5]. *Bacillus* sp isolated from sediments and seaweed had antimicrobial properties [6]. As the antimicrobial action of many microbial bioactive compounds is still un-explored, researchers are increasingly targeting it in search of rapid, novel, and effective treatments [7]. In the present study, the red alga *Acanthophora spicifera* associated heterotrophic bacteria is screened for potential antimicrobial activity, mass cultured and microbial extract was tested against XDR pathogenic bacteria collected from diabetic foot ulcers.

2. Materials and methods

2.1 Study area and algal collection

The red alga *Acanthophora spicifera* were collected from CSMCRI, Mandapam area (latitude 9°16'14"N; longitude 79°7'10"E), nearby Rameswaram, located in the Gulf of Mannar zone along the South East Coast of India. The seaweed was collected from infested rocky substratum using metal scraper and aseptically transported in ice packs to the laboratory during the month of January in the study year.

2.2 Isolation of algal associated marine bacteria

2.2.1 Isolation of epiphytic organisms

The marine algae were washed with sterilized ocean water for a few seconds to remove the superficial debris and other settled organisms. An algal portion weighing 60 g (wet weight) was cut having a 100 cm^2 (10 cm×10 cm) algal surface area. Epiphytes on the algal surface were brushed into sterile saline, and after appropriate dilutions were plated out on Zobell Marine Agar (2216e, Hi media) [8]. Incubations were done for visible colony appearance and the total epiphytic bacterial count was noted as Colony Forming Unit (CFU)/cm^2 of sample using the formula.

$$\text{Colony forming unit} = \frac{\text{CFU}\times\text{Dilution factor}}{100\ \text{cm}^2} \quad (1)$$

2.2.2 Isolation of endophytic organisms

One gram of above saline washed alcohol dipped, water washed seaweed sample was crushed in mortar and pestle with 1 mL of sterile saline water and filter using 0.45 µm pore size 'Whatmann' filter paper. The filtrate was enumerated for endophytes on Zobell marine agar plates (2216e Hi-media) using pour plate technique and expressed as CFU/g sample.

2.3 Isolation of predominant diabetes foot ulcer (DFU) associated pathogens

The pus swabs were collected from patients in a tertiary care hospital before antiseptic application from neuropathic foot ulcers of the pus were collected in duplicates from wound centre after debridement. Pathogens were enumerated on basic and specific media. Single colonies were picked, subcultured and identified using microscopic, cultural and biochemical methods**.**

2.4 Antibiotic susceptibility test to identify MDR, XDR pathogens of DFU

Following CLSI guidelines [9], the test pathogens were swabbed onto sterile Mueller Hinton agar plates and antibiotic discs were placed using sterile forceps. A 20 mm gap between 2 discs and 15 mm from disc to edge of the plate was maintained. After overnight incubation, the inhibition zone around discs is measured in mm and to the standard chart for analyzing the sensitivity pattern. Twenty antibiotics were selected based on local sensitivity pattern and pathogen type to study the antibiogram profile of pathogens. They are ampicillin, piperacillin, teicoplanin, cefuroxime, cefotaxime, cefepime, imipenem, aztreonam, gentamicin, cotrimoxazole, chloramphenicol, erythromycin, daptomycin, linezolid, ciprofloxacin, doxycyclin, tigecyclin, nitrofurantoin, polymyxin B, pristinomycin. The pathogen specific MAR (multiple antibiotic resistance) index value [10] and the maximum inhibitory percentage (MIP) of antibiotics were calculated. Based on the guidelines of European Centre for Disease Prevention and Control (ECDC), MDR, XDR, PDR classifications are done. As per definition, MDRs are pathogens showing resistance to three or more antimicrobial classes, XDRs exhibit resistance to most

antimicrobials and PDRs show 100% resistance to all antibiotics. For this study, pathogens showing resistance to more than 50% of tested antibiotics are considered XDRs.

$$\text{MAR \% (Multiple Antibiotic Resistance)} = \frac{\text{Number of antibiotics}}{\text{Total number of antibiotics}} \times 100 \quad (2)$$

$$\text{MIP \% (Maximum Inhibitory Potential of an antibiotics)} = \frac{\text{Number of susceptible organisms}}{\text{Total number of tested organisms}} \times 100 \quad (3)$$

Selective numbers from each isolated pathogens are subjected to antibiogram test and the strain from each pathogen type showing maximum MAR% are selected for further studies.

2.5 Biofilm detection assay by Congo red agar media

The biofilm producing ability of pathogens were checked on Modified Congo red agar (MCRA) plates [11] after 24 to 48 h of incubation at 37°C and observed for any blackening.

2.6 Screening assay

2.6.1 Primary screening of marine bacterial isolates by dot plate assay

A loopful of overnight log culture of marine bacterial strains were placed as separate dots on pathogen lawn inoculated onto Muller Hinton agar (MHA) plate and incubated at 37°C for 24 h. Experiment was repeated for each test pathogen. The plates were observed for clearing areas at inoculated spots. A negative control plate with un-inoculated culture broth was also maintained.

2.6.2 Secondary screening for potential marine isolate by cross streak assay

The marine isolates screened by the above method were single streaked (4-6 mm thick) in the center of MHA plates. On obtaining a ribbon-like growth of marine isolate, a loopful (0.01 mL) of overnight broth culture of MDR strains adjusted to 0.5 McFarland standard, were streaked perpendicular to it at equal distances and incubated at 37±2°C for 24 h. Five replications were made [12]. The inhibition area between marine isolate and each pathogen was measured in mm.

2.7 Mass cultivation and extraction for bioactive assays

2.7.1 Mass cultivation

The most antagonistic marine bacterial isolate for tested pathogens was mass cultivated in Zobell marine broth medium. Initially a loopful of bacterial colony was suspended in 100 mL sterile nutrient broth vortexed and adjusted to an OD value of 1 to get a final concentration of 10^6 cells /mL. Incubated at 28°C. A 10 mL of overnight bacterial broth was seeded into a 1000 mL fresh broth medium and again OD value adjusted to 1 and incubated at 28°C for 48 h in a shaking incubator (150 rpm, Lab Tech make).

2.7.2 Extraction of extracellular bioactive compound from marine bacteria

Ethyl acetate, a polar aprotic solvent, non-nucleophilic in nature, was used for the extraction of extracellular bioactive compounds from marine bacterial culture broth. The culture broth filtrate was mixed with equal volume of ethyl acetate (v/v) in separating funnel and shaken well to extract bioactive compounds and allowed to stand undisturbed for 15 min. The collected upper solvent phase was Whatmann filtered with pore size 0.45 µm diameter 4.5 cm using a Buchner funnel with suction pressure to remove bacterial cell components. This filtered extract was concentrated in a vacuum evaporator (Yamato) at room temperature for 24 h and the crude extract powder obtained was collected in air-tight plastic vials after weighing and stored in the refrigerator until further bioactivity studies [13].

2.7.3 Biofilm inhibition assay - tube method

An aliquot of 100 µL of pathogen broth culture was dispensed into test tubes containing 3 mL of marine bacterial extract prepared in 3 different concentrations in nutrient broth (10 mg/L, 100 mg/L and 1000 mg/L). No addition was made in control tube. After 48 h incubation at 37°C, the tubes were emptied and washed with 3 mL phosphate buffer saline, dried and stained for 5 min using 3 mL of 2% crystal violet solution. After gentle washing

under running tap water, observed for purple visual film. A 1.5 mL of 33% glacial acetic acid was added, mixed thoroughly and optical density (OD) was taken at 570 nm [14] against control blank.

2.7.4 Minimum inhibitory concentration (MIC)

For MIC test, the extract dilutions ranging 0.125-256 µg/mL in dimethyl sulfoxide (DMSO) were taken 5 mL aliquots in different test tubes. A 10 µL overnight MDR culture suspension was inoculated into dilutions that made $5x10^5$ CFU/mL [9]. A negative control tube received no inoculation. The antibiotic with high MIP% calculated above served the internal reference standard. The experiment included 5 replications. The tubes were incubated at 37°C for 20 h. The lowest concentration of the extract that did not permit any visible bacterial growth was recorded as MIC and compared with MIC of internal antibiotic standard for validating the inferences.

2.7.5 Minimum bactericidal concentration (MBC)

To avoid mis-interpretations due to turbidity of insoluble compounds and to determine MBC, the above tubes were plated out on homologous agar medium and enumerated to determine viable CFU/mL. A loopful of inoculum (0.01 mL) from no growth MIC tube, was subcultured on MHA plates to determine the number of surviving cells (CFU/mL) at 24 h incubation period. MBC was regarded as the lowest concentration of extract yielding a 99.9% reduction in the initial colony count of the pathogenic strain. When the ratio of MBC/MIC is ≤ 2, the active bio compounds were considered as bactericidal or otherwise as bacteriostatic [15]. A ratio ≥ 16 imply an ineffective nature of bio-compounds.

2.7.6 Anti-Inflammatory assays

2.7.6.1 Proteinase inhibitory activity

Proteinase inhibitory activity was performed according to the method of Gunathilake et al. [16]. 1 mL of 0.8% (w/v) casein was added to 2 mL of pre incubated (5 min) reaction solution (0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 mL test extract. The solution was incubated at 37°C for 20 min. At the end of incubation, 2 mL of 70% perchloric acid was added to terminate the reaction. The mixture was centrifuged, and the absorbance of the supernatant was measured at 210 nm against buffer as the blank. Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ Inhibition of denaturation} = 100 \times \frac{(1-A2)}{A1} \quad (4)$$

where A1 = absorption of the control sample, and A2 = absorption of the test sample.

2.7.6.2 Lipoxygenase inhibition assay

Lipoxygenase inhibition activity of the bacterial extract was assayed according to the method of Gunathilake et al. [16] with modifications. One mL of sodium borate buffer (0.1 M, pH 8.8) and 10 µL of lipoxygenase was incubated with 10 mL test extract at concentrations ranging 10, 20, 30, 40, 50, 60, 70 and 80% of crude at room temperature for 5 min. The reaction was initiated by the addition of 10 µL linoleic acid substrate (10 mM) and after 5 min 1% (w/v) sodium lauryl sulfate was added to terminate the reaction. The absorbance of the reaction solution was measured at 234 nm. Phosphate buffer solution was used as the control, and the percentage inhibition of lipoxygenase was calculated using the following Equation:

$$\% \text{ inhibition} = \frac{100\times(\text{absorbance of control}-\text{absorbance of the sample})}{\text{absorance of the control}} \quad (5)$$

2.7.7 GC-MS Analysis

Identification of bioactive compounds in fractionated extracts of potential anti-pathogenic bacterial strain was made and analyzed using gas chromatography coupled with mass spectrometry detection technique. 1 µL of sample was injected into an RT ×-5 column (30×0.32 nm) of GC-MS model (Perkin Elmer, Clarus 500, USA). Helium (3 mL/min) was used as a carrier gas. The following temperature gradient program was used; 75°C for 2 min followed by an increase from 75 to 175°C at a rate of 50°C per min and finally 7 min at 175°C. The m/z peaks representing mass to charge ratio characteristics of the fractions were identified by comparing with those in the mass spectrum library of the corresponding organic compounds [17].

3. Results

3.1 Isolation of endophytic and epiphytic bacteria

In the enumeration study of *Acanthophora spicifera* (Figure 1) for associated heterotrophic marine organisms, an epiphytic bacterial count of 3.6×10^4 CFU/100 cm^2 and endophytic bacterial count of 1.4×10^2 CFU/g was got on Zobell marine agar medium. All the bacterial isolates were differentiated based on macro and micro morphological characters into 28 groups comprising 15 epiphytes from algal surface and 13 endophytes (Table 1) from algal tissue homogenate. They were labeled as (EPA-01 to EPA-15) and (ENA-16 to ENA-28) respectively. Gram's staining revealed 16 Gram positives and 12 Gram negatives.

Figure 1 *Acanthophora spicifera* (red alga).

Table 1 Colony morphology of marine microbes from seaweeds.

Marine isolate Nos.	Colony morphology	Marine isolate Nos.	Colony morphology
1	Orange color round shape colony	15	Light dark color
2	White color pinpoint colony	16	Colorless colony
3	Black round shape colony	17	White small circular colonies
4	White circular colony	18	Yellow round shape colony
5	Pale yellow filamentous	19	Yellow round shape colony
6	Yellow small colonies	20	Sandal color round
7	White circular colony	21	Round filamentous like colony
8	White rough colony	22	Pale yellow color colony
9	Yellow round shape colonies	23	White Colorless colony
10	Orange color round shape colony	24	Light orange color round colony
11	White color circular colony	25	Light yellow color round colony
12	White color pinpoint colony	26	Orange round shape colony
13	Yellow color concave	27	White circular colonies
14	White rough colony	28	Pale yellow round shape colony

3.2 DFU test pathogens

The pus swab culture plates showed mixed type organisms in all the samples with Gram negative pathogens predominanting (60%). In the 30 clinical samples tested 6 clinical pathogens namely *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa, Enterococcus faecalis, Proteus mirabilis* and *Klebsiella pneumoniae* occurred in 22 (73%), 21 (70%), 18 (66%), 16 (53%), 13 (43%) and 10 (30%) numbers. Seven samples were predominated either by *S. aureus* or *P. aeruginosa* and *E. coli* predominated in 5 samples. Predominance of *E. faecalis and P. mirabilis* in 4 samples and *K. pneumoniae* in 3 samples was observed.

3.3 Results of antibiogram assay

Three isolates representing each of six pathogen genus were typed for antibiogram pattern using 20 antibiotics (Figure 2; Table 2). The MAR% of tested pathogens ranged from 55-70%. Based on ECDC definition, 83% of tested pathogens were MDRs (MAR% $\geq$ 40), and 15% were found XDRs (MAR% $\geq$ 70) which included at least one test pathogen genus. However, no organisms were found to be PDRs with 100% resistant to all tested antimicrobials. XDR pathogens were selected for further experiments.

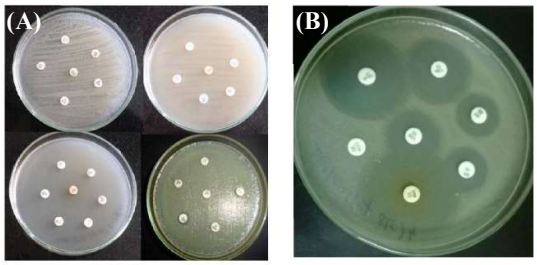

Figure 2 Antibiogram showing multiple antibiotic resistance of pathogens (A) and Sensitivity to most antibiotics (B).

Table 2 Antibiogram profile of DFU pathogens.

Antibiotics	*Pseudomonas*	*Enterococci*	*Staphylococci*	*Escherichia*	*Proteus*	*Klebsiella*	MIP%
Amplcillin	- - -	S S S	S S S	- - -	S S S	S S S	(12) 67
Piper./tazobactam	S S S	S S S	S - S	- - -	S S S	S S S	(14) 78
Teicoplanin	- - -	- - -	- - -	S S S	- - -	- - -	(03) 17
Cefuroxime	- - -	- - -	- - -	S S -	S S -	S S -	(06) 33
Cefotaxime	S S S	- - -	- - -	S S -	S S -	S S -	(09) 50
Cefepime	- - -	- - -	S - S	S - -	S S -	- - -	(05) 28
Imipenem	S S S	- - S	S - -	S S S	- - -	S S S	(11) 61
Aztreonam	S S S	- - -	- - -	S - -	S S S	S S -	(9) 50
Gentamicin	- S S	S S -	- - -	S S S	S S -	- - -	(09) 50
Cotrimoxazole	- - -	- - -	S S S	S - -	S - -	S S S	(08) 44
Chloramphenicol	- - -	S S -	- - -	S S -	S - -	S S -	(07) 39
Erythromycin	- - -	S S -	S S S	- - -	- - -	- - -	(05) 28
Daptomycin	- - S	S S S	- - -	- - -	S S -	- - -	(06) 33
Linezolid	- S S	S S -	S - S	- S -	S S -	- - -	(09) 50
Ciprofloxacin	S S S	S S S	S S S	S S S	S S S	S S S	(18) 100
Doxycyclin	- - -	- S -	S - -	- S -	- - -	- S -	(04) 22
Tigecyclin	- - -	- - -	S S S	S - S	S S S	S S S	(11) 61
Nitrofurantoin	- - -	S S -	S - -	S S -	- - -	- S -	(06) 33
Polymyxin B	S S S	- - -	- - -	S S S	S S S	S - -	(10) 56
Pristinomycin	- - -	S S S	S S S	S - -	- - -	S - -	(08) 44
Resistant antibiotics	14 12 11	10 9 14	8 14 11	6 9 14	6 8 14	7 9 14	
MAR (%)	70 60 55	70	70	70	70	70	

$$\text{MAR \% (Multiple Antibiotic Resistant \% of Pathogen)} = \frac{No.of\ antibiotics\ to\ which\ the\ org.is\ resistant}{\text{Total no.of antibiotics tested}} \times 100 \quad (6)$$

$$\text{MIP\% (Maximum Inhibitory Potential of antibiotic)} = \left(\frac{\text{No.of susceptible orgs.}}{\text{Total orgs.tested}} \times 100\right) \quad (7)$$

'S' implies Sensitive to the antibiotic; '-' implies Resistant to the antibiotic. Interpretations done as per CLSI guidelines.

3.4 Biofilming assay results

E. faecalis typed in this study was found to be a strong biofilm producer followed by *P. aeruginosa* in Congo red plate with blackening around the colonies (Figure 3).

Figure 3 Congo red plate method showing biofilming activity of pathogens.

3.5 Potential strain screening using antagonism bioassay (dot plate and cross streak assay)

In the present study red algal associated marine bacterial isolates were tested for antipathogenic property against DFU XDR pathogens using Dot plate (Figure 4) and Cross streak method (Figure 5; Table 3).

Figure 4 Dot plate assay: Clearing spots in pathogen lawn showing growth inhibition at marine culture inoculation spots (A) in lab, (B) against natural background light.

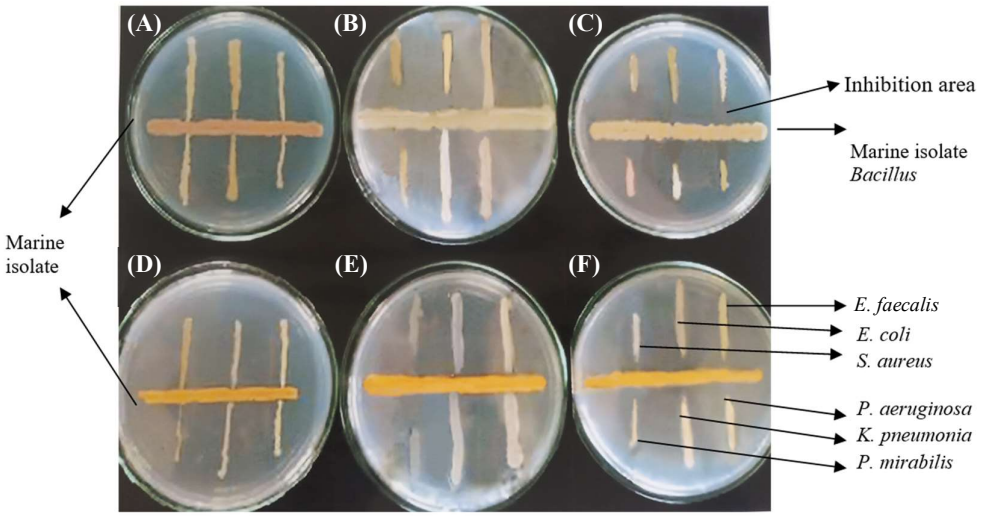

Figure 5 Cross streak assay showing marine isolate antagonism against pathogens:(A) EPA-10, (B) EPA-11, (C) EPA-12, (D) ENA -20, (E) EPA-07, and (F) EPA-04.

Table 3 Cross streak assay result - Pathogen inhibition of marine isolates.

Marine strains	Inhibition distance from marine streak (mm)					
	S. aureus	*E. faecalis*	*K. pneumoniae*	*E. coli*	*P. mirabilis*	*P. aeruginosa*
EPA-12	20±1.8***	19±1.5***	16±1.0***	16±1.0***	15±1***	15±1***
EPA- 04	17±1.1***	13±1.2	7±1.5	11±1.1***	7±0.6	8±0.5
EPA- 07	14±1.4***	4±1.8	-	4±0.7	17±2.6***	-
EPA -11	17±1.1***	-	-	14±0.2***	11±1***	-
ENA-19	6±1.3	7±1.6	4±0.5	4±0.3	-	4±0.7
ENA-22	-	4±0.5	6±0.1	7±1.5	-	4±0.2
ENA-16	-	5±1.2	-	4±0.3	6±1.2	4±0.6
EPA- 08	-	5±1.1	-	5±1.2	3±0.4	-
ENA-26	-	4±0.3	6±1.2	7±1.4	5±1.1	4±0.6
EPA- 03	7±1.7	4±0.3	4±0.6	-	6±1.3	5±1.4

N=5, p <0.05, ± values are std. deviations, *** indicates highly significant difference in Post hoc t- test.

Of all the 28 marine isolates, 18 of them did not show any growth clearance or inhibition of pathogens in Dot plate assay or Cross streak assay respectively. But 10 of them have shown clearing areas at applied spot in Dot plate assay and produced growth inhibition distances in Cross streak assay, measured in mm. Of this, a Gram positive epiphytic isolate (EPA-12) showed significantly high (p >0.05) inhibition activity against all tested pathogens and was selected for further mass culturing. In Cross streak assay, maximum inhibition by EPA-12 was noted ranging from 15-20 mm between marine streak and test pathogens when compared to other marine groups. Marine isolates, EPA - 4 & 11 showed 17 mm inhibition area only for *S. aureus* and EPA - 7 could control Proteus

with 17 mm inhibition. Other recorded inhibitions fell in the 4 - 8 mm range only. *Staphylococcus and Enterococci* were maximum inhibited (20±1.8 mm, 19±1.2 mm) by EPA-12, followed by *Escherichia coli* and *Klebsiella* (16±1mm). The potential strain was motile, aerobic, spore forming, starch hydrolyzing, catalase positive, VP negative, growing at 6.5% NaCl, producing acid from arabinose and suggested to be *Bacillus* sp. (*Bacillus circulans)*. EPA-12 marine isolate that controlled all the XDR test pathogens with inhibition distances ≥ 15 mm was alone chosen for mass culturing.

3.6 Extraction of bioactive compound and its antibacterial effect

From mass culturing system, a 300 mL of 48 h old broth culture of EPA-12 were treated with ethyl acetate. A final 1.5 g of crude extract powder obtained in DMSO suspension was used for further assays.

3.6.1 Results of biofilm inhibition assay

The marine bacterial crude extract exhibited a similar effective inhibition of biofilm formation by the 2 tested pathogens (*E. faecalis* and *P. aeruginosa*). A strong inhibition was observed at 1000 mg/L concentration followed next by 500 mg/L concentration, but 100 mg/L concentration was found inefficient in controlling biofilm formation by both pathogens (Figure 6).

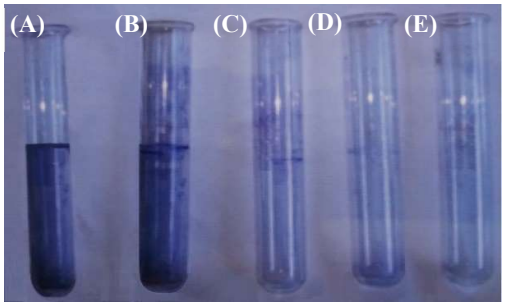

Figure 6 Biofilm inhibition in treatments with *E. faecalis*; (A) positive control, (B) 100 mg/L extract, (C) 500 gm/L extract, (D) 1000 mg/L extract, and (E) negative control.

3.6.2 Results of MIC and MBC test

Ciprofloxacin with 100% pathogenic inhibition was used as reference antibiotic. It inhibited Gram negative and positive pathogens at 16 µg/mL and 32 µg/mL concentrations respectively. The test extract could control pathogens at 64 µg/mL and 128 µg/mL i.e.., twice and eight times the concentration of ciprofloxacin respectively (Table 4). The MBC/MIC ratio was found to be ≤ 2 in all cases thus validating and assuring result reliability for bactericidal property.

Table 4 MIC results of bacterial extract and Ciproflaxacin antibiotic standard for DFU.

Test pathogens	Bio-active test compound MIC (µg/mL)	Ciproflaxacin MIC (µg/mL)
E. coli	128^{c}	16^{d}
P. aeruginosa	128^{c}	16^{d}
K. pneumoniae	128^{b}	16^{e}
P. mirabilis	128^{b}	16e
S. aureus	64^{a}	32^{b}
E. faecalis	64^{a}	32^{b}

N=3, Mean comparison done by t-test. Different alphanumerical represent highly significant differences between means in rows and columns (p <0.05).

3.6.3. Results of anti-inflammation assays - proteinase and lipoxygenase inhibition

Proteinase inhibitory activity of test extract was found to be 58% when compared to control. Inhibition levels were within the range of 15-67.0% within the concentrations tested. The extract at 70% concentration showed an improved ability to inhibit proteinase activity (about 67.0%), whereas 10% concentration has shown the least inhibitory activity (15.0%). Lipoxygenase inhibitory activity of test extract was found to be 44% when compared to control. Inhibition levels were within the range of 12-53.0% within the concentrations tested. The extract at

70% concentration showed an improved ability to inhibit lipoxygenase activity (about 53.0%), whereas 10% concentration has shown the least inhibitory activity (12.0%) (Figure 7).

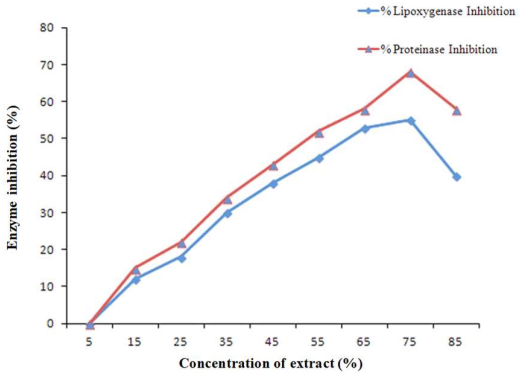

Figure 7 Proteinase and Lipoxygenase inhibitory activity of bacterial extract. Values represent means of triplicate readings.

3.7 GC-MS analysis of isolated endophytic bacterial bioactive compound

The list of GC-MS analyzed compound of crude extract of potential EPA-12 isolate is given in Figure 8 and Table 5.

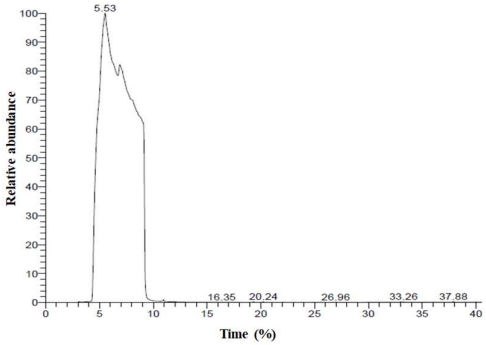

Figure 8 GC-MS chromatogram of potential EPA-12 crude extract.

Table 5 GC-MS analysis showing bioactive compounds of marine strain crude extract.

S.No.	Peak value	Compound name	Molecular formula	Molecular weight	Applications
1.	1.47	Cyclohexanol, 2-methyl-, acetate, trans-	C9H16O2	156	Antibacterial, Antifungal activity
2.	1.53	3-Ethoxy- 4-(trimethylsilyl) cyclobut-3-ene-1,2-dione	C9H14O3Si	198	Pharmaceutical intermediates
3.	1.90	(E)-1-[trimethylsilyl]hex-1-ene	C9H20Si	156	Antibacterial and Anticancer activity
4.	2.06	2,4,6-Tripropyl-1,3,5-trioxane	C12H24O3	216	Antibacterial activity
5.	2.33	4-Trimethylsilylfuran-2(5H)-one	C7H12O2Si	156	Anti-inflammatory activity
6.	3.31	Ethyl 2,2,4,4-tetramethyl-5-oxo-3-thiahexanoate	C11H20O3S	232	Pesticidal and Insecticidal
7.	3.59	R)-1,1-Dimethoxy-2-propanamine	C5H13NO2	119	Antibacterial activity
8.	3.74	S)-1,1-Dimethoxy-2-propanamine	C5H13NO2	119	Antibacterial activity
9.	4.05	3-(2-Dimethoxymethyl-5-hexenoyl)-2-oxazolidinone	C12H19NO5	257	Antioxidant
10.	4.35	(E)-1,5-undecadien-3-yne	C11H16	148	Pharmaceutical and insecticidal
11.	4.53	9-Oxa-is (1,8), cis (2,7), trans (7,8)-tricyclo [6.3.0,0(2,7)] undeca-3,5,10-triene	C10H10O	146	Antioxidant and Antibacterial activity
12.	4.53	cis and trans-,3,6-Dicetoxy-1-cyclohexene	C10H14O4	198	Antitumor, antifungal activity
13.	5.36	9-Oxa-cis (1,8), cis (2,7), trans (7,8) – tricyclo [6.3.0,0(2,7)] undeca-3,5,10-triene	C14H14O	198	Antioxidant and Antibacterial activity
14.	5.53	6,7-Bis(3,4-dimethoxyphenyl)-1,2,3-trihydroindolizine-5-one	C24H25N O5	407	Antimicrobial activity

4. Discussion

Marine habitat's complex network of bio-association constitutes a sizeable reservoir of novel antimicrobial compounds, exhibiting anti-inflammatory activity [18]. They become the source of new lead for treating many infectious diseases. Though antimicrobial activity is widespread among algal associated bacteria, their characterization is still at an early stage of development. Bioactive compounds formed by such associations may have profound use in the ailment of diabetic foot ulcers caused by microbial infection.

A good load of microbes in the tested swabs of ulcer sites suggested that the inflammations observed were a consequel of polymicrobial infection process. *Staphylococci* followed by *Escherichia* and *Pseudomonas* were found the most prevalent, and *Klebsiella* the least prevalent [19] causative agents as reported in previous studies. The predominance and prevalence percentage of pathogens differed among samples. *Escherichia* was more prevalent but, in less counts, than Pseudomonas. Patient's immunity and defense status, and the pathogenic association prevailing in the infection site may influence the microbial load and microbial types in foot ulcers. The high MAR index noted in the study reveals the possible health risk due to antibiotic resistant pathogens.

Most of the algal associated isolates with high antimicrobial and antifouling activity were reported as *Bacillus* species that remain as highly successful colonizers of macroalgal surfaces [20] The potential antimicrobial activity of red algal associated marine *Bacillus* sp. of the present study can be attributed to its mutualistic relationship with the host or a natural means to overcome competitive exclusion. In mutualism epibiotic bacterial community derives nutrients and physical protection from host and in turn protects it from biofouling.

Inability of bacterial cells from MIC wells to resume growth on the MHA agar indicated the bactericidal nature of antimicrobials. Ciprofloxacin standard, being a purified active ingredient, had lower MICs than that of crude algal epiphytic extract. MIC results revealed the need to use higher concentrations of crude extract with much purification to attain a similar antibacterial activity as that of the standard. In the previous studies made by Nurul et al. [5] *Acanthophora* ethyl acetate extracts showed a moderate average zone of inhibition that ranged from 9.00-14.00 mm. The MIC and MBC values of selected bacterial strains tested ranged from 31.25-1000 µg/mL and 500-2000 µg/mL^{-1}. The flavonoids and proanthocyanidins in algal extracts could have significantly contributed to the antibacterial properties. In the present study the ethyl acetate extract of same algal associated potential bacilli was found more effective with inhibition zones ranging upto 20 mm and MIC and MBC values ranging 62-128 µg/mL^{-1}for similar pathogens of above study. This showed that marine algal associated bacteria possessed higher antibacterial activity than the alga itself, since the bacterial extract composition is different consisting of many varied components with antibacterial and antimicrobial effects.

GC-MS listed compounds were of both polar and non polar nature as evident from the functional groups like amines, hydroxyl, keto or alkyl groups. Many researchers had reported non polar lipid soluble extracts from marine macroalgae to have antibacterial activity against Gram positive and Gram negative pathogenic strains [21]. Bactericidal antimicrobials can diffuse freely through lipid membranes to bring upon cell death. While the present study extract demonstrated a fairly good activity against Gram negative pathogens, it is against Gram positive pathogens that it proved to be more potent with lower MIC. These variations might be due to genetic or biochemical composition of pathogenic bacteria.

For patients with diabetes, it is a must to control ulcers to prevent amputations for which ulcer management includes control of inflammation and infection. Bacterial proteases can lead to necrosis and apoptosis of phagocytes and cause death of normal tissue cells [22]. Phagocyte dependent growth factors required for cellular proliferation and various healing components are impaired by proteases of infecting microbes and delay diabetic wound healing. These proteases must be neutralized and stopped from being produced. The proteinase inhibition level was higher for present study algal epiphytic extract when compared to phytoextracts as in earlier studies by Gunathilake et al. [16].

Compounds that disrupt the arachidonic acid metabolism by blocking lipoxygenase activity and scavenge various reactive free radicals formed during the process can help in preventing inflammatory responses in host. The extracts from red alga *Odonthalia corymbifera exhibit* lipoxygenase inhibitory activity probably through molecular interactions inside the enzymes' active sites [23]. In the present study also the red algal epiphytic extract has shown high lipoxygenase inhibitory activity. A dip in lipoxygenase activity after 70% concentration may be due to other components in extracts that might interfere antagonistically with lipoxygenase inhibition activity.

Earlier studies have demonstrated the antibiotic activity from macroalgae due to the presence of organic acids, methyl acetates and unsaturated fatty acids similar to present study. The presence of halogen substituted organic compounds in study extract can be related to their bioactivity, bioavailability, and stability. The major novel component in GC-MS analysis in this study was found to be oxazolidinones. Derivatives of oxazolidinones are useful as antibacterial [24], anti allergy and anti-inflammatory agents (United States Patent 5208250). The next predominant GC-MS compound propionates are considered anti-inflammatory [25]. Additionally, indolizine ring inclusion should support anti-inflammatory activity with other promising biological properties such as antioxidant, analgesic and antibacterial activities [26]. The trioxane compound is reported antibacterial [27]. Another compound 4-Trimethylsilylfuran-2(5H)-one was reported to display anti-inflammatory, anti-pyritic,

analgesic properties with suppression of cyclooxygenase enzymes. Furanones interfere with Quorum sensing processes [28].

A cost effective unharmful drug with dual role of anti-inflammatory and anti-bacterial property can be a better therapeutic. Presence of indolizine rings, silylfuranone, oxazolidinone derivatives in the algal epiphyte Bacillus sp. EPA-12 extract should contribute for antibacterial, anti-inflammation, QS disruption and proteinase impairment properties and can serve a source for drug lead for treating diseases and infections.

5. Conclusion

Bactericidal biocompounds with anti-inflammatory properties are the immediate constructive demands to manage the diabetic foot ulcers effectively. The associative epiphytic bacteria of marine red seaweed *Acanthophora* can act as a good source of lead compounds towards DFU treatment. Crude extract bioactive components from the screened marine epiphytic bacteria were found to have bactericidal activity and anti-biofilming activity against DFU pathogens. GC-MS analysis of extract revealed many anti-microbial and anti-inflammatory molecules which can be of therapeutic value for diabetic ulcers. Further scope of this study involves purification processes and toxicity studies which can improve the safety and application efficiency of test compounds.

6. Ethical approval

The collection of swab samples was in accordance with ethical guidelines and approved by Government Mohan Kumaramangalam Medical College and Hospital, Salem, Tamil Nadu - Institutional Ethics Committee (IEC approval No.: GMKMCH/2623/IEC/01/2016-78).

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