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Antibacterial activity of *Jania rubens* from Gulf of Mannar, south coast of India

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Seaweeds are primarily explored for pharmaceutical and functional applications. This is due to the bioactive properties such as antimicrobial, antiviral, antitumor, anticancer and antilipidemic beneficial for such industries. Most of the countries surrounded by coastal regions have indicated a growth of research on seaweeds to utilize them in drug development. The present study was conducted to investigate the *in vitro* antibacterial activity of ethanol extract of *Jania rubens* against bacterial pathogens such as *Escherichia coli, Klebsiella pneumoniae*, *Staphylococcus aureus, Salmonella typhi, Streptococcus pyogenes, Enterococcus faecalis, Pseudomonas aeruginosa, Bacillus subtilis and Proteus vulgaris*. It is a slender, rose pink, articulated, calcified fronds, in ordered bunches to 50 mm high. Epiphytic, only found growing epiphytically on older plants of the brown algae. The antibacterial activity was analysed by the agar disc diffusion method. The ethanol extract of *J. rubens* was separated based on activity directed fractionation to isolate the principle bioactive compound. The fraction was purified under Thin layer chromatography and characterized by Gas chromatography and Mass spectrometry. The mean zone of inhibition produced by the ethanol extract in disc diffusion assay against bacterial strains ranged from 9.0 to 26 mm. The highest mean zone of inhibition (26.00±0.56 mm) was observed against *E. faecalis*. The extract was further separated using Water, n-hexane and Ethanol. The fraction showed the antibacterial activity was characterized as 5-(Hydroxymethyl)-2-(1-methyl-2-2imidazolyl)-1H-benzimidazole predominantly by GC-MS.

Keywords: Antibacterial activity, Gas Chromatography–Mass Spectroscopy, Gulf of Mannar, *Jania rubens*, Seaweed, TLC.

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Introduction

Seaweeds are macroalgae, which generally reside in the littoral zone and can be of many different shapes, sizes, colours, and compositions. They include brown algae, red algae and green algae. They have a highly variable composition, depending on the species, time of collection and habitat, and on external conditions such as water temperature, light intensity and nutrient concentration in water[1]. The algal extracts were used as a curative and preventive agent for various diseases such as antibiotics, anti helminthics, cough remedies, antihypertensive, antitumor, and antidiarrhoea. Recently we have embarked on the chemical investigation of marine algae with a special accent on their bioactive properties. About 2400 natural products have been isolated from seaweeds[2-3]. Harder (1917) was the first to observe antimicrobial substances secreted by algae[4]. The phytochemicals from marine algae are extensively used in various industries such as food, confectionery, textile, pharmaceutical, dairy and paper, mostly as gelling, stabilizing and thickening agents. In addition to proteins, vitamins and minerals, seaweeds are also potentially good sources of polysaccharides and fibres[5-6]. Seaweeds contain both water and fat-soluble vitamins such as B1, B2, B6, B12, vitamin C and A, D, E, K[7]. The carotenoids, including carotene, fucoxanthin and chlorophyll, present in the majority of seaweeds, act as antimutagens, antioxidants or antipromoters for cancer[8-9]. *Jania rubens* is a calcified red alga from 15 to 40 mm high, has a rose-red colouration, although in strongly illuminated areas this can be slightly yellowish-white. It grows slender rose-pink fronds that form rounded bunches reaching 5 cm long. It has a thallus formed from cylindrical filaments; the fronds are erect and jointed with particularly thin branches. *J. rubens* belongs to red algae classified under *Florideophyceae*, subclass in *Corallinophycidae*, order is *Coralliinales* and the family is *Corallinaceae*. This alga is fixed by a small conical disc but spreads vegetatively by developing attachment discs from branches in contact with solid substrate. It has maximum development during autumn and winter. The

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ramification is dichotomous with the branches tapering near the end; swellings found at the joints are the reproductive bodies. Branches are up to 2.5 cm long, occasionally with secondary pinnate (feathery) branching, giving the alga a luxurious, fluffy look. *J. rubens* is rather rare and is found in well-lit, subtidal rocky surfaces from 8 to 10 m deep, in sandy sea floors and herbariums of eelgrass. It can grow either directly attached to the rock by small conical discs (where it can spread by forming new attachment points on branches in contact with substrate) or attached to other algae (epiphytically). It is typically found as an epiphyte on brown and red macro-algae. *J. rubens* is used by the cosmetic industry as an extract in natural beauty products. It is prized for its ultra-moisturizing and protective properties due to the high concentration of minerals and trace elements present in its tissue. Soliman *et al.*, evidenced the analgesic, antipyretic and anti-inflammatory effects exhibited by the brown seaweed *J. rubens*[10]. Paraguerene and isoparguerene from *J. rubens* was isolated[11]. Solvent extract of *J. rubens* was proved in controlling the growth of *Vibrio fluvialis*[12]. *J. rubens* is characterized by a concentration of minerals and traces elements 20,000 to 40,000 times greater than that of seawater, thus giving it remineralizing properties. It is also used in skin whitening and hydrating products. This alga helps to regulate the expression of slimming genes and enables to slow down the synthesis and the storage of fats. The crude extract thus obtained is subjected to broad biological screening for antifungal, antiviral, antibacterial, antimalarial, antifilarial, hypoglycemic and anti-fertility activity. The present study was performed with the marine seaweed *J. rubens* to investigate the preliminary *in vitro* antibacterial activity against human pathogens and to reveal the bioactive constituents in the ethanol extract using GC–MS analysis.

Materials and Methods

Collection of seaweeds

The seaweed was collected from Pudumadam, Mandapam coast, East of India, Rameshwaram (Lat 9º 16'N; Long 78º 69'E) by handpicking. (Fig. 1-2). The collected seaweed was cleaned well with seawater to remove all the extraneous matter such as epiphytes, sand particles, pebbles and shells and brought to the laboratory in plastic bags. The sample was then thoroughly washed with freshwater, blotted and spread out at room temperature for drying. The seaweed was shade dried and grounded to a fine powder. The powdered seaweed was stored under refrigeration for further use.

Fig. 1 — Collection of seaweeds from the Mandapam coast.

Fig. 2 — *Jania. rubens*.

Herbarium preparation

For the morphological identification of the collected seaweed, the whole part of the fresh Seaweed was placed between the multiple layers of filter paper and bundled tightly until the seaweed to get complete dryness to avoid fungal contamination. The filter paper was changed with the interval of every three days. The seaweeds were then removed and pasted on chart paper (24 cm X 43 cm) using the gum.

Identification of seaweeds

The seaweed was identified and authenticated by Dr V. Veeragurunathan, Scientist, Marine Algal Research Station, Central Salt and Marine Research Institute (Council of Scientific and Industrial Research), Ramanathapuram District, Tamil Nadu, India.

Extraction of seaweed

Fifty grams of dried *J. rubens* mixed with 112 mL of absolute ethanol was kept in magnetic stirrer at 1400 rpm for 1 week. The mixture was filtered and concentrated using Rotary vacuum evaporator. The concentrated crude extract was refrigerated at 4 °C until tested[13].

Microbial strains

Bacterial strains used for the assay were as following: *Escherichia coli, Klebsiella pneumonia* (M11), *Staphylococcus aureus* (M12), *Salmonella typhi* (M13), *Streptococcus pyogenes* (B11), *Enterococcus faecalis* (B12), *Pseudomonas aeruginosa* (M14), *Bacillus subtilus* (M15) and *Proteus vulgaris* (M16). Bacterial strains were obtained from Bioline laboratories, Coimbatore and Kovai Medical Center and Hospitals, Coimbatore and identified according to standard procedure (Bergey's Manual, Fungal identification Manual).

Antibacterial activity using disc diffusion method[4]

The antimicrobial activities were carried by the disc diffusion method. The antibacterial assay against pathogenic bacteria was carried out using the agar plate method. The bacterial inoculum was grown in nutrient broth overnight and a fixed volume was inoculated onto Muller Hinton agar by swabbing. This formed the bacterial lawn. The paper disc of 6 mm in diameter was loaded with 50 µL of crude extract and placed onto the bacterial lawn. Ampicillin (10 µg/mL) was used as the positive control. After 24 h of the incubation period, the plates were observed for the antimicrobial activity exhibited by the extract[14].

Antibacterial activity directed isolation of functional compounds

The low or medium polar fractions usually contain lipophilic organic compounds and the high polar fractions contain water-soluble compounds[15]. Bioassay is used to localize the active component in the partitioning process, being considered as a preliminary step to isolate an active compound. The bioactive constituents present in the ethanol extract of *J. rubens* were further separated and purified based on the antibacterial activity spectrum. The crude extract was further fractionated using water, n-hexane and ethanol respectively (Fig. 3). On each fractionation process, the collected fractions were tested by the agar disc diffusion method against the Gram-positive organisms (Table 1-3). Based on the spectrum of antibacterial activity, the fraction was taken for further separation (Fig. 4-5). The final fraction showed the activity was taken for purification using thin-layer chromatography and the bioactive compound present in the fraction was identified using Gas chromatography and Mass spectrometry analysis. The antibacterial assay was followed by sequential gradient partitioning, which will help in the chromatographic separation of the compounds according to their polarity. Exactly 100 g of the dried sample was soaked in 1 L of absolute ethanol for a

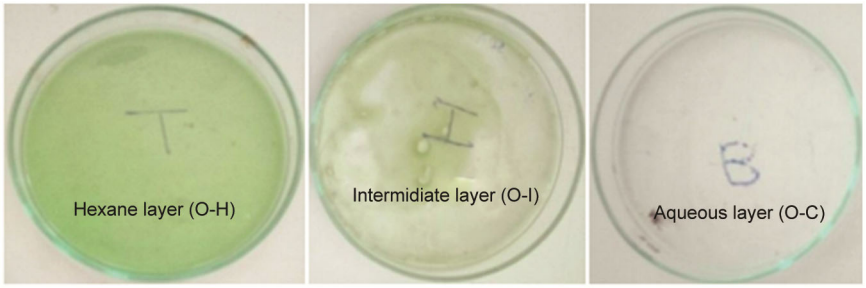

Fig. 3 — Hexane and Aqueous fractions collected from separating funnel.

Table 1 — Antibacterial activity spectrum of crude extract, Fraction O–H, O–I and O–C against the selected gram-positive bacteria

Name of the extract	Zone of inhibition (mm)*		
Layer	*S. aureus*	*S. pyogens*	*E. feacalis*
Crude	-	12.6±0.5	25±0.15
Hexane (O-H)	-	12.3±0.15	25.2±0.8
Intermediate (O-I)	-	13.8±0.6	12.1±0.1
Aqueous (O-C)	-	8.1±0.3	10.2±0.2

-: No visible antibacterial activity was observed

*The value indicates the Standard Error of Mean of experiments done in triplicates.

Table 2 — Antibacterial spectrum of ethanol soluble (O-HA) and ethanol insoluble (O-HB) fractions

Test Strains	Zone of inhibition (mm)*	
	Ethanol Soluble (O–HA)	Ethanol Insoluble (O-HB)
S. aureus	11.3±0.05	-
S. pyogens	10.2±0.15	-
E. faecalis	23.2±0.05	-

Table 3 — Antibacterial spectrum of ethanol soluble (O-HA) fraction from TLC

Test Strain	Zone of inhibition (mm)* for compound separated on TLC (O–HA1)
S. aureus	10±0.06
S. pyogens	-
E. faecalis	22.2±0.3

*The value indicates the standard Error Mean of experiments done in triplicates.

week. The sample was continuously stirred in a magnetic stirrer at a constant speed (1400 rpm) during the period and then filtered. The filtrate was dried on a rotary evaporator at 40 °C. The dried extract was mixed and shaken thoroughly with 400 mL of n-Hexane and 400 mL of distilled water in a 1L separating funnel and the contents were left till complete separation of the layers. Three different layers were collected separately as fraction 'O-H' (n-Hexane – upper, green layer), fraction 'O-W' (aqueous – lower, yellow layer) and fraction 'O-I' (Middle solid insoluble mass). The crude ethanolic extract and the fractions O-H, O-I, O-W were screened for their antibacterial activity against *E. faecalis.* The fraction showed antibacterial activity was further partitioned into two portions ethanol-soluble (O-HA) and ethanol insoluble (O-HB). The screening was repeated, the fraction retains the activity was dried and then analysed by TLC-silica using chloroform – acetic acid (9:1) as mobile phase. The developed plate was dried under normal air and the spots were visualized under UV dark chamber at 254 & 365 nm. The separated bands were scratched from the plates and dissolved in ethanol – acetone (1:1). The extracts were dried and analysed for antibacterial activity.

Characterisation of the functional compound using GCMS

The purified fraction of *J. rubens* was identified using Gas Chromatography-Mass Spectrophotometer (GC-MS) in DB 35-MS capillary standard non-polar column at a temperature between 70 °C - at 6 °C per minute. The sample was run using helium as carrier gas at the flow rate of 1.0 mL/min. The compound was identified based on the database of National Institute Standard and Technology NIST4 and WILEY9.

Results and Discussion

Collection of seaweed

The seaweed was collected from Mandapam coastal area, East coast of India and the Geographical location is given. Synthetic drugs are very expensive, inadequate, often with adulteration and side effects. These create a need to explore new strategies to control microbial infections[16]. Numerous reports for the compounds derived from seaweed with a broad range of biological activities, like antimicrobial[17,18-19], antiviral[20], neurotoxin, antitumors and anti-inflammatory[21].

Screening of antibacterial activity using disc diffusion assay

The zone of inhibition was measured and tabulated (Table 4). The ethanolic extract of *J. rubens* showed

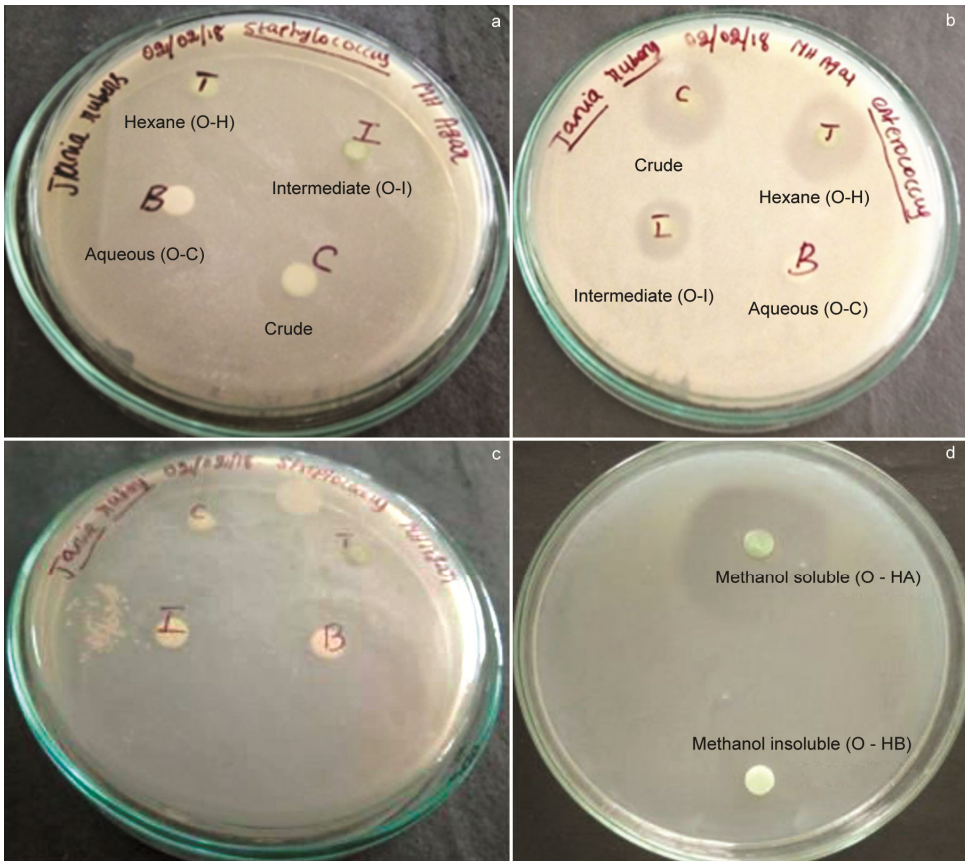

Fig. 4 — a) Antibacterial activity of crude extract, Fraction O–H, O–I and against *S. aureus*, b) Antibacterial activity of crude extract, Fraction O–H, O–I and O–C against *E. faecalis*, c) Antibacterial activity of crude extract, Fraction O–H, O–I and against *S. pyogens* and, d) Antibacterial activity of Ethanol soluble (O-HA) and Ethanol Insoluble (O-HB) against *E. faecalis*.

Fig. 5 — Spot on TLC.

Table 4 — Antibacterial activity of *J. rubens* against the selected pathogens of ethanol fraction

Bacterial pathogens	Diameter of zone of inhibition (mm)*
E. coli	09.00±0.81
E. faecalis	26.00±0.56
S. aureus	10.00±0.54
P. aeruginosa	11.00±0.41
S. pyogenes	22.00±0.75
B. cereus	10.00±0.22
P. vulgaris	10.00±0.88
K. pneumonia	11.00±0.41
S. typhi	10.00±0.48
Amphicillin	17.00±0.02

good activity against all the tested bacterial species. The extract showed a maximum mean zone of inhibition against *E. faecalis* (26.00±0.56 mm), *S. pyogenes* (22.00±0.75 mm), and followed by *S. aureus* (10.00±0.54 mm). However, the extract showed only moderate activity against all the tested Gram-negative bacterial strains (9.00±0.81 mm to 11.00±0.88 mm). The results of the present study were harmonizing to the previous report gave by

Taskin[22] *et al.*, stated the extracts of seaweeds showed more inhibition on Gram-positive bacteria than the Gram-negative bacteria due to the differences in their cell wall structure and their composition[23-24]. Several studies have focused on the antimicrobial properties of seaweed extracts[25,26-27]. The findings by Vallinayagam[28] *et al.,* stated that the red algae showed maximum activity than the green and brown algae when tested against human pathogens. Sujatha[29] *et al.*, reported the highest antibacterial activity of *J. rubens* against *Staphylococcus aureus*. They also evidenced maximum biochemical compounds in ethanol extract rather than aqueous extract. Mohamed[30] *et al.*, reported that *Klebsiella pneumoniae* was the most sensitive microorganism to *J. rubens* extract. In a similar study, it is reported that *J. rubens was* active only against Gram-positive bacteria while no activity was recorded against Gram-negative one[31]. From the above findings, the resistance of gram-negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharides molecules, presenting a barrier to the penetration of numerous antibiotic molecules. The membrane is also associated with the enzymes in the periplasmic space which are capable of breaking down the molecules introduced from outside[32]. The antimicrobial activity was previously reported by Val[32] *et* al., that the moderate activity were observed in the red seaweed *J. rubens* against the growth of the bacteria *P. aeruginosa, S. marcescens, E. faecium, M. smegmatis, S. aureus* and *B. subtilis*. The study by Karabay[33] *et al.*, has reported that the ethanolic and chloroform extracts of *J. rubens* had significant antimicrobial activity[11]. A few of the previous studies recorded high antimicrobial activity in the aqueous extract of *J. rubens* against *Bacillus subtilis* and low activity against *S. aureus*[10]. The earlier research observed higher activity against Rhodophyta was in line with that toluene-ethanol (1:3) extracts of species belonging to Rhodophyceae exhibited broad-spectrum activity when compared to Chlorophyceae and Phaeophyceae[34].

Separation of bioactive constituents using activity directed fractionation method

The gradient partitioning of the seaweed extracts showed the activity in the ethyl acetate phase, indicating the non-polar nature of active fractions[35]. The activity observed in non-polar fraction could be attributed to the presence of lipophilic compounds such as terpenoids, acetogenins and compounds of mixed biosynthesis that occur relatively in low concentration (ranging from 0.2 to 2% of algal dry weight)[36-38]. In the present study, the hexane layer (O-H) showed effective antibacterial activity than the intermediate layer (O-I) and aqueous layer of the extract. The hexane fraction was again extracted with ethanol. Among the fraction tested, only ethanol-soluble fraction showed inhibition on bacterial strains. The fraction was further purified using thin-layer chromatography. The single spot present in thin layer chromatography was collected and extracted with ethanol using centrifugation. The ethanol-soluble fraction showed good inhibition against the tested bacterial strains.

Identification of active metabolite using GC-MS

The nature of this purified antibacterial compound was detected using GC-MS (Fig. 6). According to the stated data, the antibacterial compound present in *J. rubens* was identified as 5(Hydroxymethyl)-2–(1-methyl-2-imidazolyl-1H-benzimidazole). The chemical formula was $C_{12}H_{12}N_4O$. Molecular weight was 228 which was characterized at the retention period of 20.30 with the probability of 92.01 as a major component (Table 5). Benzimidazoles have been reported to have antimicrobial properties against bacteria or fungi. The azetidinone ring bearing compounds showed varied biological activities like antibacterial and antifungal activities[39].

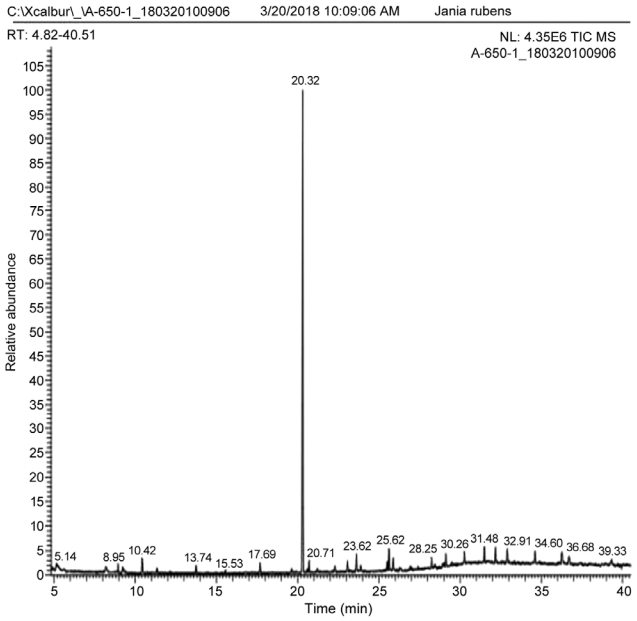

Fig. 6 — Identification of active metabolites using GC-MS.

Table 5 — Chemical constituents of *J. rubens* ethanol extract as investigated by GC-MS

S. No.	Compounds	R. time	Probability	Mol. Formula	Mol. Wt.	Percentage
1	Benzene 1,3,5 trimethyl	5.18	13.74	C_9H_{12}	120	1.38
2	2(3H) – Benzofuranone	8.95	20.15	$C_{11}H_{17}DO$	166	0.75
3	Ethyl – Hexahydroxypyrrolo – quinoline	10.44	28.61	$C_{14}H_{19}N$	201	0.91
4	Cyclohexy (2-methylenecyclohexy) ethanol	13.74	47.05	$C_{14}H_{24}O$	208	0.71
5	Heptadecane	17.69	8.55	$C_{17}H_{36}$	240	0.91
6	5(Hydroxymethyl) -2 – (1-methyl-2-imidazolyl-1H-benzimidazole)	20.30	92.01	$C_{12}H_{12}N_4O$	228	48.35
7	2-Pentadecanone	20.71	60.17	$C_{18}H_{36}O$	268	1.20
8	Hexadecenoic acid	23.62	52.92	$C_{18}H_{36}O_2$	284	1.54
9	Octadecenoic acid	25.62	14.90	$C_{19}H_{36}O_2$	296	2.70
10	Isopropenyl – 1,4A-Dimethyl-Hexahydro-Naphthalenone	28.25	11.53	$C_{15}H_{21}DO$	218	1.54
11	Dotriacontane	30.26	11.38	$C_{32}H_{66}$	450	1.16
12	Tricosane	31.48	11.12	$C_{23}H_{44}$	324	1.50
13	Nonacosane	32.91	12.57	$C_{29}H_{60}$	408	1.66
14	Hexatriacontane	36.70	11.07	$C_{36}H_{74}$	506	1.38

Conclusion

The spectrum revealed the antibacterial compound present in *J. rubens* was 5(Hydroxymethyl) -2 – (1-methyl-2-imidazolyl-1H-benzimidazole). The chemical formula was $C_{12}H_{12}N_4O$. Molecular weight is 228 was characterized at the retention period of 20.30 at the probability of 92.01 as major component. The study concluded that the ethanol extract of *J. rubens* belonging to the Rhodophyta group from Mandapam Coast used in the present investigation possesses the highest inhibition against all the tested human bacterial pathogens thus promising a future scope for the use of this marine seaweed against microbial populations.

Conflict of interest

The authors declare no conflict of interest.

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