

Original Article

**PHYTOCHEMICAL EVALUATION AND ANTIMICROBIAL ACTIVITY OF *GRACILARIA OPUNTIA*: AN IMPORTANT ANTI-DIABETIC RED MARINE MACROALGAE**

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ABSTRACT

**Objective:** Microorganisms have developed resistance to existing available antibiotics, thereby thriving to an emerging need for new generation of antibiotics. Since seaweeds provide a rich source of bioactive molecules, the present study aimed to investigate its anti-microbial potential against clinically important microorganism.

**Methods:** Red sea weed namely *Gracilaria opuntia* collected from different coastal regions of Gulf of Mannar and Rameswaram, India was used. For microbiological testing of the seaweed extracts, agar disc diffusion method was used.

**Results:** The zone of inhibition was measured for all the different crude algal extracts against strains of microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Pseudomonas putida* that cause diseases in diabetic patients. Crude extracts prepared from aqueous, ethanol and methanol extraction procedures revealed that aqueous extraction procedure have a wide range of antimicrobial activity against all the test pathogens.

**Conclusion:** The overall antibacterial activity assessed from the above results indicates the presence of active constituents in the extractions of seaweeds which can be explored for the production of significant molecules which could be used in pharmaceutical industry.

**Keywords:** *Gracilaria opuntia*, Antimicrobial activity, Agar diffusion method, Solvent extracts

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INTRODUCTION

The sea, covering 70% of the Earth's surface, offers a considerably broader spectrum of biological diversity than terra firma. Containing approximately 75% of all living organisms, the marine environment offers a rich source of natural products with the potential therapeutic application [1]. A report suggests that marine organisms are source material for structurally unique natural products with pharmacological and biological activities [2-4].

Among the marine organisms, the macroalgae (seaweeds) occupy an important place as a source of biomedical compounds [5-6]. Seaweeds are the eukaryotic organism that lives in salty water in the ocean and it is found to be a potential source of bioactive natural products [7]. They contain compounds including sterols, terpenoids, phenolic compounds which show bioactive against microorganisms.

In recent years, there are numerous reports of macro algae-derived compounds that have a broad range of biological activities such as antibacterial, antifungal, antiviral, antineoplastic, antidiabetic, anti-inflammatory, anti-tumoric, cytotoxic and antimutagenic activities [8-9]. About 2400 natural products have been isolated from macroalgae belonging to the classes Rhodophyceae, Phaeophyceae and Chlorophyceae [10]. Presently seaweeds constitute commercially important marine renewable resources which are providing valuable ideas for the development of new drugs against diabetes, microbial infections and inflammations [11]. Algal constituents include acids, alkaloids, amines, antibacterial, antifungal, antiviral substances, lipids, sterols, steroids, fatty acids, phenolic compounds, phytochromes, pigments, proteins, peptides, amino acids, sugar, alcohols and vitamins. *Gracilaria opuntia* belongs to the family Rhodophyceae (Red algae). These are highly evolved multicellular forms with well-developed branched thalli. Except for few species, they are exclusively marine and vary in size and shape. They are epiphytes, growing as a crust on the rocks or shells as a large fleshy, branched or blade-like thalli. The thallus is basically filamentous, simple or branched, free or compacted to form pseudoparenchyma

with uni or multiaxial construction. They inhabit intertidal to subtidal zones of coastal areas [12].

The present study was aimed to study the phytochemical screening and antimicrobial activity of *Gracilaria opuntia* solvent extracts against pathogenic bacteria.

MATERIALS AND METHODS

Collection of marine algae samples

The identities of the red marine macroalgae *Gracilaria opuntia* considered in the present study were ascertained with the sample specimens maintained in the Marine Biodiversity Museum of Central Marine Fisheries Research Institute. The marine macroalgae were collected freshly from the Gulf of Mannar in Mandapam region located between 8°48' N, 78°9' E and 9°14' N, 79°14' E on the south-east coast of India during the months spanning between August-April. Samples collected (2 kg) were washed in running water and shade dried before being pulverized to a minimum particle size.

Preparation of marine macroalgae extracts

The powdered marine macroalgae samples (1000 g) were extracted with *n*-hexane (600 ml x 2), at room temperature for 24 h, and the pigments were separated. The residue was filtered through Whatman No.1 filter paper and extracted three times with methanol (MeOH) and ethanol (EtOH) (50–60 °C, 3 h), respectively, filtered through Whatman No. 1 filter paper, and the obtained filtrate was concentrated at 50 °C in rotary vacuum evaporator (Heidolf, Germany) to get the dark brown viscous mass of MeOH (112 g) and EtOH fractions (96 g), respectively. The aqueous extracts of the seaweed were prepared by extracting the dried marine macroalgae powder (500 g) with 80–90 °C hot water for 3–4 h. The contents were thereafter cooled (4 °C) and centrifuged at 8500 rpm for 15 min (Sorvell Biofuge Stratos, Thermo Scientific, USA) to remove the solid residues that were freeze-dried to get the crude aqueous extract (27 g). The aqueous extract of *G. opuntia* was concentrated

before precipitated with alcohol (500 ml). The precipitate was lyophilized to get a dried oligosaccharide fraction of *G. opuntia*. This was then powdered and packed in vacuum packed bags and stored in the refrigerator until further use.

#### Phytochemical screening

Phytochemical analysis of all the evaporated solvent extracts was conducted following the procedure of Indian pharmacopoeia [13].

#### Test for alkaloids

200 mg of plant material was dissolved in 10 ml of methanol and filtered. For 2 ml filtrate and 1% HCl+steam, 1 ml filtrate+6 drops Mayer's reagent/Wagner's reagent/Dragendorff's reagent was added. Creamish precipitate/brownish red precipitate/orange precipitate indicated the presence of respective alkaloids.

#### Test for tannins

200 mg of plant material was dissolved in 10 ml of distilled water and filtered. For 2 ml filtrate+2 ml FeCl<sub>3</sub> was added. Blue/black precipitate indicated the presence of tannins.

#### Test for flavonoids

200 mg of plant material was dissolved in 10 ml of ethanol and filtered. For 2 ml filtrate+conc. HCl+magnesium was added. Ribbon pink/tomato red color indicated the presence of flavonoids.

#### Test for steroids (Liebermann-burchard reaction)

200 mg of plant material was dissolved in 10 ml of chloroform and filtered. For 2 ml of filtrate+2 ml of acetic anhydride+H<sub>2</sub>SO<sub>4</sub> was added. Blue/greenring indicated the presence of steroids.

#### Test for phenols

1 ml of each solvent extracts dissolved in alcohol/water was separately treated with 1 ml of neutral FeCl<sub>3</sub>. The change in colour indicated the presence of phenols.

#### Test for sugars

The extract (100 mg) was dissolved in 5 ml of water and filtered. To 2 ml of filtrate, two drops of an alcoholic solution of  $\alpha$ -naphthol were added, the mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. Violet rings indicate the presence of carbohydrates.

#### Test for saponins

The extract (50 mg) was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. A 2 cm layer of foam indicates the presence of Saponins.

#### Antimicrobial screening

##### Laboratory test organisms and their cultural conditions

The test bacterial strains used for the study were *Escherichia coli* (ATCC 9837, Gram-negative), *Staphylococcus aureus* (ATCC 6538, Gram-positive), *Klebsiella pneumoniae* (ATCC 1705, Gram-negative) and *Pseudomonas putida* (ATCC 12633, Gram-negative). Specific growth conditions were maintained. The bacteria used for the study were procured from ATCC. All the cultures were maintained at 4 °C in nutrient agar slants.

##### Preparation of inoculums

A loop full of the test culture was taken from respective strains of agar slants and subcultured in fresh tubes containing nutrient broth and incubated for overnight at 37 °C. The obtained cultures were centrifuged at 5000 rpm for 15 min. Bacterial suspension was added to fresh media which gives a final concentration of 10<sup>7</sup> cfu/ml [14].

##### Antibacterial activity assay

The antibacterial activity was tested by agar well diffusion method [15] as adopted earlier and with little modifications was used [16]. Seeded agar was made using nutrient agar medium. After the medium preparation, it was sterilized and allowed to cool so that the

medium gets solidified. Just before solidification 0.1 ml of diluted inoculum (10<sup>5</sup> cfu/ml) of test organism was added to the medium and then it was poured into the sterilized petri dishes under aseptic conditions. Under sterile conditions, wells of 4 mm diameter were punched into the agar medium with the help of sterile cork borer. These wells were filled with 10, 25, 50, 75 and 100  $\mu$ l of algae extract and of 500  $\mu$ g/ml concentration and solvent Dimethyl sulphonylate (DMSO) as a control. The plates were incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the zone of inhibition (in mm) against test organisms. The antibiotics ampicillin, tetracycline and erythromycin at 500  $\mu$ g/ml concentration each were used as positive controls.

## RESULTS AND DISCUSSION

### Phytochemical analysis

Preliminary investigation of phytochemical analysis of selected crude extracts revealed the presence of various compounds such as alkaloids, flavonoids, phenols, saponins, steroids, sugars and tannins. Alkaloids, flavonoids detected in the extracts are compounds that have been documented to possess a variety of medicinal properties and health-promoting effects. Phenolics are the largest group of phytochemicals have been said to account for most of the antioxidant activity of algae extract. These classes (such as alkaloids, flavonoids, phenols, saponins, steroids, sugars and tannins) of compounds are known to have curative activity against several pathogens and therefore could suggest the use traditionally for the treatment of various illnesses [17]. From the results depicted in table 1 higher concentration of alkaloids were present in aqueous extract and maximum concentrations of flavonoids were present in aqueous compared to ethanol and methanol extracts. Higher concentration of phenols was recorded in ethanol and methanol extracts when compared to aqueous extracts. The moderate concentration of saponins was observed in aqueous, ethanol and methanol extracts of *G. opuntia*. The three extracts don't shows any steroid. High-level sugars were present in the aqueous, ethanol and methanol extracts. Moderate concentrations of tannins were present in aqueous and methanol extracts compared to ethanol extracts. The most of the phytochemicals classified as secondary metabolites are produce from algae, often their function unknown, but certain phytochemicals have structural, functional and general defence against pathogens so the preliminary phytochemical studies received pronounced importance, because the crude drugs possess varied composition of secondary metabolites [18]. Considering the high economical and pharmacological importance of secondary metabolites, industries are deeply interested in the large-scale production of algal substances [19-20].

### Anti-microbial activity

Seaweeds are the eukaryotic organisms that live in salty water in the ocean and is recognized as a potential source of bioactive natural products [7]. They contain compounds ranging from sterols, terpenoids, phenols which may help to enhance the antimicrobial activity of red marine algae [8]. In the present study, the antibacterial activity of three different solvents viz., aqueous, methanol and ethanol extracts of *Gracilaria opuntia* was evaluated against pathogenic bacteria. Among three solvent extracts tested, the aqueous extract showed the greatest inhibition diameters against gram positive and gram negative bacterial isolates (fig. 1 and 2 and table. 2). The similar results were observed *G. folifera* and *G. carrasa* [21, 22]. The results from the present study showed that the gram-positive bacteria are more susceptible than gram negative bacteria on seaweed extracts which was also supported from earlier works with different species of seaweeds indicating that the more susceptibility of gram-positive bacteria to the algal extracts was due to the differences in their cell wall structure and their composition [23].

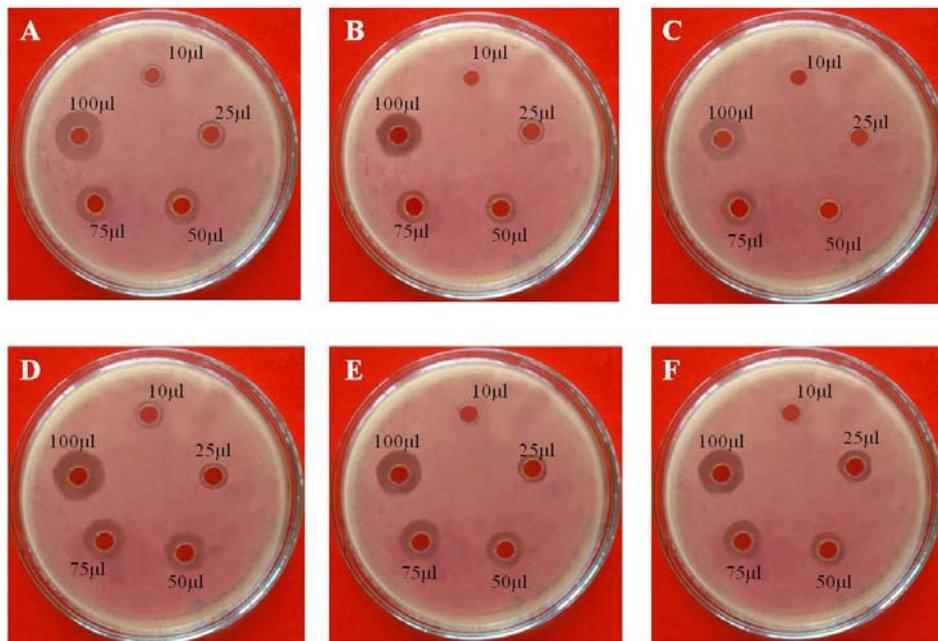
The aqueous methanol and ethanol extract of *Gracilaria opuntia* at a different concentration such as 10, 25, 50, 75 and 100  $\mu$ g/ml showed a zone of inhibition. Among them, the aqueous extract has showed the highest zone of inhibition (4.8 mm) against the *E. coli* followed by *Pseudomonas putida* (4.6 mm), *Klebsiella pneumoniae* (4.5 mm), and *Staphylococcus aureus* (4.0 mm) at 100  $\mu$ g/ml concentration. The minimum zone of inhibition was recorded in methanol and ethanol

extract of *Gracilaria opuntia* against *E. coli* (3.5 and 3 mm) followed by *Pseudomonas putida* (2.5 and 2.3 mm), *Klebsiellapneumonia* (4.3 and 3.5 mm), and *Staphylococcus aureus* (3.5 and 1.5 mm).

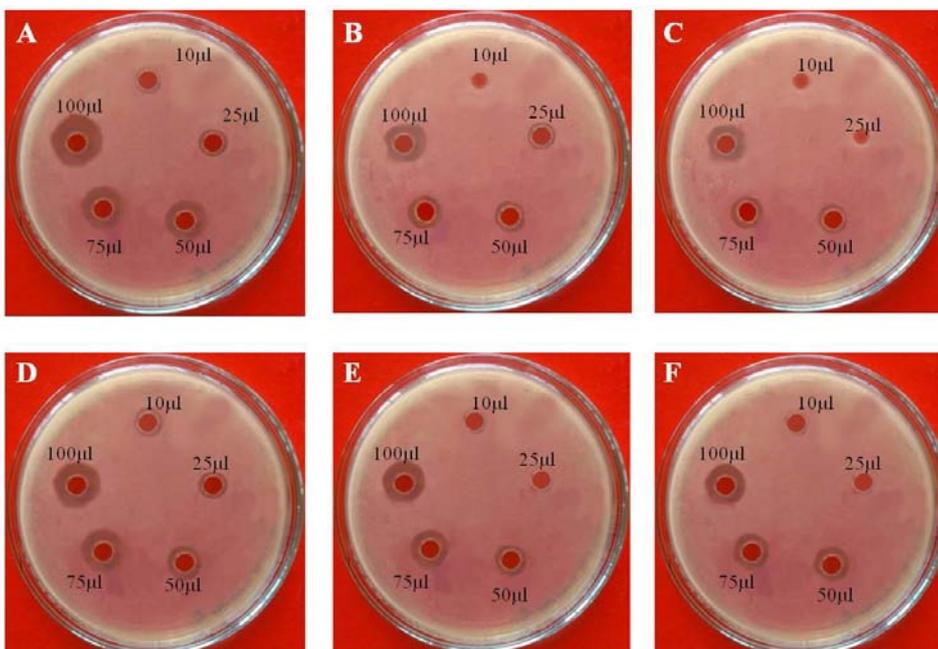
The zone of inhibition obtained from the aqueous extract of seaweed *Gracilaria opuntia* against bacterial pathogens was comparatively very high when compared to the methanolic and ethanolic solvent extracts. No zone of inhibition was seen in DMSO control and the positive control ampicillin, tetracycline and erythromycin showed a zone of inhibition ranging from 6.0 mm to 8.2 mm against the test bacterial pathogens (fig. 3).

Invariably, seaweeds have been proven to be a potent source of antimicrobial compounds. Aqueous extract of *Gracilaria opuntia* belongs to Rhodophyta exhibited broad-spectrum antibacterial activity [24].

Previously it has been reported in some of the marine macroalgae extracts such as *Enteromorpha compressa*, *Cladophora siphonoides*, *Padinagymnospora*, *Sargassum wightii* and *Gracilaria corticata* were active against gram positive and gram negative bacteria [25]. Based on the present findings, it could be inferred that the bioassay-guided fractionation and purification may come up with potent antibacterial compounds.



**Fig. 1: Antibacterial activity of aqueous (A, D), ethanol (B, E) and methanol (C, F) extracts of marine macro algae-*Gracilaria opuntia* against *Escherichia coli* (A, B, C) and *Klebsiellapneumonia* (D, E, F). Upon treating with *G. opuntia* at different concentrations the antimicrobial activity is reduced. 100 µl has shown a significant zone of inhibition**



**Fig. 2: Antibacterial activity of aqueous (A, D), ethanol (B, E) and methanol (C, F) extracts of marine macro algae-*Gracilaria opuntia* against *Pseudomonas putida* (A, B, C) and *Staphylococcus aureus* (D, E, F). Upon treating with FM4 at different concentrations the antimicrobial activity is reduced. 100 µl has shown a significant zone of inhibition**

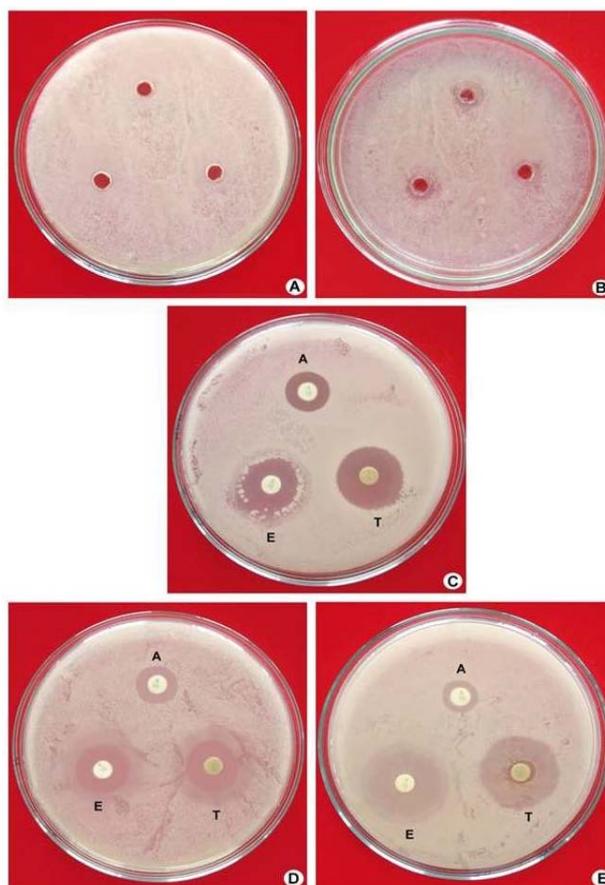


Fig. 3: A-B) DMSO used as a negative control to test the activity against *Pseudomonas putida* and *Staphylococcus aureus*. C-E) Commercial antibiotics such as tetracycline (T), ampicillin (A) and erythromycin (E) used as a positive control against *E. coli*, *S. aureus* and *P. putida*

Table 1: Phytochemical analysis of secondary metabolites such as alkaloids, flavonoids, phenols, saponins, steroids, sugars and tannins in *Gracilaria opuntia*

Type of secondary metabolites	Aqueous extract					Methanolic extract					Ethanollic extract				
Alkaloids	++					+					+				
Flavonoids	++					+					+				
Phenols	-					+					+				
Saponins	+					+					+				
Steroids	-					-					-				
Sugars	++					++					++				
Tannins	+					+					-				

Table 2: Antimicrobial activity of aqueous, ethanolic and methanolic extracts of marine macroalgae *Gracilaria opuntia*. Among the three extracts, aqueous extract has shown significant and maximum zone of inhibition compared to ethanolic and methanolic extracts treated groups

S. No.	EXTRACTS- $\mu$ l micro organisms	<i>Gracilariaopuntia</i> (Aqueous) (Zone of inhibition in mm)					<i>Gracilariaopuntia</i> (Ethanol) (Zone of inhibition in mm)					<i>Gracilariaopuntia</i> (Methanol) (Zone of inhibition in mm)				
		10	25	50	75	100	10	25	50	75	100	10	25	50	75	100
1	<i>Escherichia coli</i>	1.2	2.0	2.7	3.0	4.8	-	1.5	2.2	2.8	3.5	-	-	0.5	1.5	3
2	<i>Klebsiella pneumoniae</i>	0.5	1.0	1.8	2.5	4.5	-	0.5	1.0	2.0	4.3	-	1.5	1.5	2.0	3.5
3	<i>Pseudomonas putida</i>	0.5	1.0	2.0	2.0	4.6	-	0.5	0.5	1.0	2.5	-	-	1.0	1.5	2.3
4	<i>Staphylococcus aureus</i>	0.5	1.0	1.5	3.0	4.0	-	-	1.0	2.0	3.5	-	-	0.9	1.1	1.5

## CONCLUSION

The present research concluded that the organic solvent extraction was suitable to verify the antimicrobial properties of *Gracilaria opuntia* and they supported by many investigations. The

investigation on antimicrobial activity of extracts of *Gracilaria opuntia* showed that the aqueous extract shows promising antimicrobial activity when compared to other solvent extracts. The results also indicated that scientific studies carried out on seaweed extracts having traditional claims of effectiveness might warrant

fruitful results. *G. opuntia* could serve as a useful source of new antimicrobial agents. The present study justifies the claimed uses of *G. opuntia* in the traditional system of medicine to treat various infectious diseases caused by the microbes. These results suggest the possibility of using marine algae extracts in therapy as natural alternatives to antibiotics currently in the market, and clearly show that seaweeds are a valuable source of biologically active compounds. Further research is underway to determine the structure and nature of these antibacterial substances.

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#### CONFLICTS OF INTERESTS

Authors declare no conflicts of interest

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