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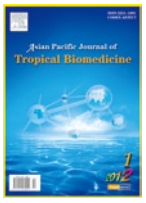

Document heading

Nuclear magnetic resonance analysis for antimicrobial compounds from the red seaweed *Gracilaria corticata*

Govindasamy C*, M. Arulpriya, P. Ruban

Department of Oceanography and Coastal Area Studies, School of Marine Sciences, Alagappa University, Thondi Campus-623 409, Tamilnadu, India

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ABSTRACT

Objective: To explore the antimicrobial compounds from the rhodophyceae family member algae *Gracilaria corticata* using ^{1}H and ^{13}C NMR spectral analysis. **Methods:** Antimicrobial activity was done by using the method of Prabhakar *et al*. Methanol and acetone extracts were tested against the human pathogens. The acetone extract was subjected to column chromatography using acetone as a solvent for purification and also the chemical analysis combined with ^{1}H and ^{13}C NMR analysis. **Results:** Among the two extracts, acetone extract showed higher activity than methanol extract. ^{1}H and ^{13}C NMR analysis showed the fraction extracted with acetone had polysaccharide like compounds. **Conclusions:** Altogether our observations suggest a triggering of antimicrobial activity by this polysaccharide like compound. Further the investigations are now in progress to purify and identify the structure of the compounds.

1. Introduction

Seaweeds are floating and submerged plants of shallow marine meadows. Marine macro algae are important ecologically and commercially to many regions of the world, especially in Asian countries. Seaweeds are potential source of bioactive metabolites for the pharmaceutical industry in drug development. Many of the seaweeds possess bio-active components which inhibit the growth of some of the Gram positive and Gram negative bacterial pathogens[1]. They are a valuable food resource which contains low calories, and they are rich in vitamins, minerals, proteins, polysaccharides, steroids and dietary fibers[2-4]. The algae synthetize a variety of compounds such as carotenoids, terpenoids, xanthophylls, chlorophyll, vitamins, saturated and polyunsaturated fatty acids, amino acids, acetogenins, antioxidants such as polyphenols, alkaloids, halogenated compounds and polysaccharides such as agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan sulfate, galactosyl glycerol and fucoidan[5-15]. Compounds with cytostatic, antiviral, antihelmintic, antifungal and antibacterial activities have been detected in green, brown and red algae[16,17]. Recent research has pointed to new opportunities, particularly in the field of medicine, associated with bioactive properties of molecules extracted from seaweeds[18]. Approximately 841 species of marine algae found in both inter-tidal and deep water regions of the Indian coast[19]. In India southeast coast is a unique marine habitat infested with diverse seaweeds. *Gracilaria* genus (Gracilariales, Rhodophyta) is a macroalgae group with more than 300 species of which 160 have been accepted taxonomically. These are usually red, green or greenish brown with a three-phase cycle and can be found in tropical and subtropical seas[20,21]. The *Gracilaria* species are important for the industrial and biotechnological uses. Therefore the present study was undertaken to evaluate the antimicrobial compounds from the macro algae *Gracilaria corticata* (*G. corticata*).

2. Materials and methods

*Corresponding author: Dr. C. Govindasamy, Associate Professor and Head, Department of Oceanography and Coastal Area Studies, School of Marine Sciences, Alagappa University, Thondi Campus- 623 409, Tamilnadu, India.
Tel: 09444256247
E- mail: govindasamyocas@gmail.com
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2.1. Algae collection and extract preparation using two different solvents

The sample was collected from the Mandapam region in the eastcoast of India. *G. corticata* was thoroughly washed with ambient seawater to remove the epiphytes and extraneous matter. Then the samples were thoroughly washed with sterile distilled water, air dried, cut into small pieces and then ground in to fine powder. The coarse powder of *G. corticata* was subjected to Soxhlet extraction separately and successively with acetone and methanol. The apparatus was run for 4 h and the syrupy extracts were collected. Finally, these extracts were concentrated to dryness in rotary evaporator under reduced pressure and controlled temperature. Both the extracts were stored in a refrigerator in air tight containers. Both the crude extracts were analyzed for preliminary antimicrobial activity.

2.2. Antimicrobial Assay and Minimum Inhibitory Concentration (MIC)

The agar disc diffusion method was followed for the susceptibility test[22,23] for the following human bacterial and yeast strains *Streptococcus pneumonia*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Candida* sps. Bacterial and yeast suspensions were prepared by inoculating one loopful of pure colony into 10 mL of sterile broth. Then, 1 mL of the suspension was pipetted into 15 mL of sterilized molten Muller Hinton agar and potato dextrose agar aseptically. The plate was then swirled well. The agar medium was left to solidify. 20 mg of each extract was dissolved in 1 mL of respective solvent. 6 mm discs were impregnated with 30 μL of the extracts and placed in the strains inoculated in the Muller Hinton agar and potato dextrose agar medium. Each disc will have exactly 60 μg of the seaweed extracts. The inoculated plates were incubated at 35 ℃ for 24-28 h and the inhibition zones measured around the discs (mm diameter). Amoxyclav (AC for *Streptococcus pneumonia*), linezolid (LZ for *Staphylococcus aureus*), netilin (NT for *Proteus mirabilis*, *Escherichia coli* and *Pseudomonas aeruginosa*) and amphotericin (AP for disc *Candida albicans* and *Candida* sp) were used as a positive control and each disc was containing the concentration of 30 μg of substance.

The MIC values were studies for the microorganisms that were susceptible in the previous screening. A serial of 2- fold dilutions of the extracts were set up using sterile Muller Hinton broth and Sabourated dextrose broth medium as dilutions in 10ml sterile test tubes containing 500 μl of the inoculum, to give final crude extract concentrations within the range of 10 to 160 μg/mL. All the tubes were incubated at 37 ℃ for 24 h for bacteria and yeast. MIC values was determined by comparing the turbidity of the whole tubes with negative control (Nutrient broth inoculated with bacteria and without extract) and two positive control test tubes (nutrient broth only and nutrient broth with the extract only). The lowest dilution of the tube that showed no visual turbidity was taken as MIC Value.

2.3. Purification of crude extracts using column chromatography

Selected active crude extracts (2 g) were fractionated by column chromatography on silica gel (60-120 mesh). Column (2 cm × 40 cm) was set up in Acetone with silica gel (30-40 g). The column fractions (100 mL each) were evaporated under vacuum. Fractions which were freed from solvent, re-dissolved in appropriate solvent and screened for activity by disc diffusion method as described above.

2.4. NMR analysis

The fraction with the highest activity (Fraction B) was taken and dissolved in NMR standard acetone and poured in 5 mm probe. For ^{13}C NMR, the frequency was 100 MHz and for ^{1}H NMR, the frequency was 400 MHz. The resultant NMR spectrum was then analysed manually.

3. Results

3.1 Antimicrobial assay and MIC

The red algae *G. corticata* exhibited broad spectrum of antibacterial activity and its extract inhibited all the tested bacteria in different activity ranges. The two extract were tested for antimicrobial activities against the Gram positive, Gram negative and yeast pathogens. Table 1 shows

Table 1.
Antimicrobial activity and Minimal inhibitory activity of *G. corticata* extracts.

Pathogens	Control(mm)	Antimicrobial assay (Inhibiting zone mm)		MIC	
		Acetone	Methanol	Acetone	Methanol
Streptococcus pneumonia	12	15	R	>50	100
Staphylococcus aureus	28	6	17	60	50
Proteus mirabilis	16	17	17	40	>50
Escherichia coli	17	12	R	>50	100
Pseudomonas aeruginosa	23	17	14	40	60
Candida albicans	10	18	15	40	>50
Candida sp.	19	16	11	40	>50

Table 2.
Antimicrobial activity of *G. corticata* acetone fractions (mm).

Pathogens	Control	GCF-1	GCF-2	GCF-3	GCF-4
Streptococcus pneumonia	13	R	15	12	13
Staphylococcus aureus	26	7	9	12	11
Proteus mirabilis	16	14	16	13	11
Escherichia coli	18	16	12	10	9
Pseudomonas aeruginosa	20	17	18	17	10
Candida albicans	8	R	14	12	8
Streptococcus pneumonia	19	11	13	R	12

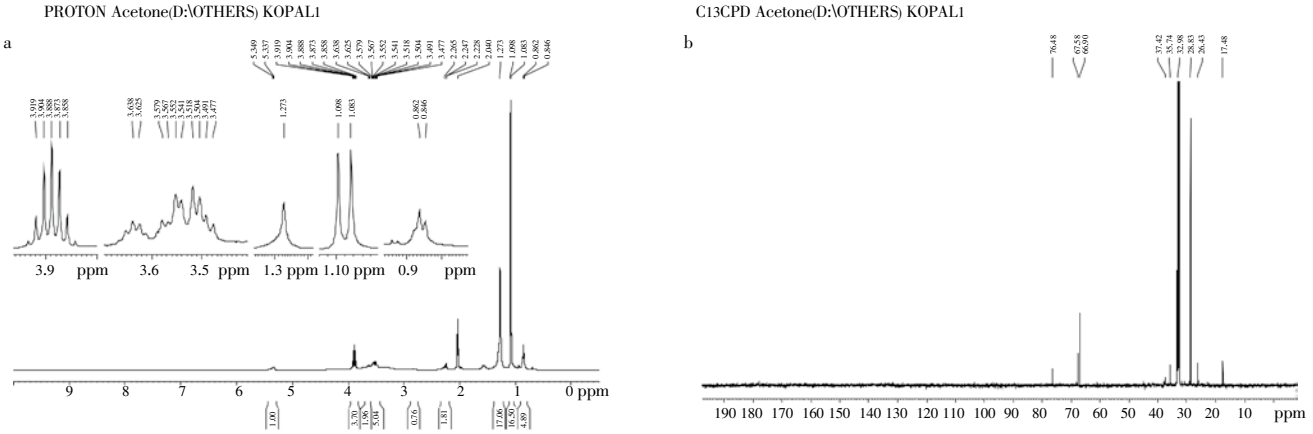

Figure 1. NMR spectral analysis of fraction GFC-2.
a and b. Showing the NMR spectral analysis of fraction GFC- 2.

the antimicrobial activity and MIC results for the extracts extracted using the soxhlet apparatus. Among the two extracts, Acetone extract was showed higher activity than methanol extract because acetone extract contain most potent antimicrobial compounds compared with methanol extract. 60 μg of acetone extract was active against gram negative bacteria, yeast and the inhibition zones were in the range of 15-18 mm in diameter. But both the extract showed less antimicrobial activity against the gram positive bacteria *(Staphylococcus aureus)*. Methanol extract was susceptible to *Streptococcus pneumonia* and *Escherichia coli*.

3.2 Antimicrobial activity of column fractions

Selected active acetone extract of *G. corticata* were fractioned by column chromatography on silica gel. The fraction GCF-2 showed more activity against the human bacterial and yeast pathogens. Maximum activity was observed against *Pseudomonas aeruginosa* (18 mm) followed by *Proteus mirabilis* (16 mm) and *Streptococcus pneumonia* (15 mm). Fraction GCF-3 showed inhibitory activity against seven human pathogens, but susceptible to *Candida* sp (Table 2). Based on the antimicrobial activity the fraction GCF-2 was taken for NMR analysis.

3.3. NMR spectrum and its analysis

The proton NMR of fraction B showed peaks of different intensities in the following regions of chemical shift (d ppm): 0.846, 0.862, 1.083, 1.098, 1.273, 2.040, 2.228, 2.247, 2.265, 3.477, 3.491, 3.504, 3.518, 3.541, 3.552, 3.567, 3.579, 3.625, 3.638, 3.858, 3.873, 3.888, 3.904, 3.919, 5.337, and 5.349 (Figure 1a). The ^{13}C NMR of fraction B showed peaks of different intensities in the following regions of chemical shift (d ppm): 17.48, 26.43, 28.83, 32.98, 35.74, 37.42, 66.90, 67.58, and 76.48 (Figure 1b). The proton NMR shows five signals. The methyl proton CH_3-C group showed its signal at δ = 0.862 ppm. The methyl proton signal is split into doublets due to the coupling of neighbouring (-CH) proton which was confirmed from the signal at δ = 1.083 ppm. The signal at δ = 1.273 ppm is attributed to methyl proton of CH_3- C-O group. The multisplet at δ = 3.541 ppm is may be due to presence of Methylene proton of -C-CH_2 group. The second multiplet at δ = 3.888 ppm is due to presence of -C-CH-OH group.

The ^{13}C NMR contains nine signals. The resonance signals at d= 17.48 ppm is attributed to the methyl group. The Methylene (-CH_2-) group is confirmed from presence of signal at d= 28.83ppm. The sharp peak at δ = 66.9 ppm is due to carbon environment of CH. The weak resonance signal at d= 76.48 ppm is may be due to alkyl group. The comparative analysis of both the spectra shows the presence of polysaccharide like compounds along with hydrolysed sugar moieties that are products of the hydrolysis of the main compound.

4. Discussion

Acetone extract showed maximum level of activity than methanol extract. Another study in methanol extract of

Gracilaria gracilis sp. showed same result as that of *G. corticata* methanol extract[24,25]. Previous studies revealed that the *G. corticata* extract with different solvents were exhibit antimicrobial activity in gram positive, gram negative and fungal species[26-31]. *G. corticata* has antibacterial, antifungal, hypoglycaemic, diuretic, antiprotozoal, antifertility and antiviral activities. It has also a marked effect on the central nervous system of experimental animals[32]. MIC concentration was minimum of 40 μg and maximum of 100 μg per mL. Three main galactan sulphates have been identified in *Gracilaria* sp. Which show antiviral activity against herpes simplex virus strains 1 and 2. The minimum inhibitory concentration was found to be 10 μg per mL[33].

The activities of the purified fractions were almost similar to the activity of the crude extracts. Among the fractions, fraction B showed maximum activity. This is probably due to the fact that the middle fraction (GCF-2) has high concentration of active products.

The NMR spectrum result of the fraction GCF-2 showed primarily polysaccharides. Oligosaccharides are commonly defined as carbohydrate molecules with a low degree of polymerization (between 2 and 25). These molecules may be found naturally or derived from larger polysaccharides[34]. This is surprising since organic extracts of seaweeds normally show secondary metabolites. In many previous studies, aqueous extracts of *G. corticulata* have shown the presence of complex polysaccharides ranging from neutral molecules to highly charged galactans[33, 35]. The ability to synthesize acid polysaccharides is the most interesting property of red algae. Several species produce complex heteropolysaccharides containing uronic acids together with neutral or sulfated monosaccharides or galactans[36]. Red algal galactans constitute a spectrum of polysaccharides encompassing a variety of non-glycosyl substitutions (methoxyl, pyruvate, sulfate groups, other sugar residues (galactose, xylose) or uronic acids[37]. Sulfated polysaccharides obtained from seaweed *G. corticata* have proven antiviral activities against many viruses[38,39]. This information, combined with the fact that the genus *Gracillaria* is an agarophyte, throws light on the result. The percentage of polysaccharides could have been so large as to mask every other compound. Also, here since we are considering only one fraction, this is also indicator of the fact that the polysaccharides could have been concentrated in fraction B.

In conclusion it is suggested that the Polysaccharides like compounds present in the fraction play an important role in the defence against the pathogens. Further the investigations are now in progress to purify and identify the structure of the compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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