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Determination of Phenol Compound and Anti-Bacterial Potential in Methanol Extract of Marine Red Algae *Gracilaria Fergusonii* J. Agardh

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Abstract

The genus *Gracilaria* is one of the important agar yielding marine macro algae belongs to Rhodophycean member. They are macroscopic multicellular thallus attached the rocky substratum of inter tidal region of seashore. The sulfated polysaccharides obtained from the cell wall of *Gracilaria* are used in pharmaceutical and nutraceutical industries. More over the genus have abundant primary bioactive constituents were reported by many workers and are still succeed to identifying active compounds in every day. The present attempt was aimed to analysis the bioactive compounds from the methanol extract of *Gracilaria fergusonii* under various spectral studies such as Fourier Transformer Infrared Spectroscopy (FTIR), UV-Visible Spectrophotometer (UV-VIS), H^1-NMR and Gas Chromatography Mass Spectrometry (GC/MS). As a results, the functional phenol group showed in all the spectral profile. Phenols are alkayl substituted aromatic ring with singlet or multiple chemical shift and are used in industrial chemical, food additive, antioxidant etc. In FTIR, the sharp peak showed range between 3300-3500cm^{-1} and in UV-Vis spectrum, the sharp peak showed at 315 nm with the absorbance of 2.459. Meanwhile, in H^1NMR the chemical shift ranges δ5.14ppm, δ 7.27 ppm, δ 7.46 ppm and δ 7.73 ppm were confirm the phenol group of tested methanol extract of *G. fergusonii*. Another confirmation found in GC/MS 13.18 RT showed a compound Phenol, 2, 4-bis (1, 1-dimethylethyl) - with molecular weight of 206 and the peak area of 0.73%. That compound has good result in the study of anti-bacterial activity against human pathogens. The biochemical composition of total free phenols also estimated from *G. fergusonii* that showed 3.08±0.022 mg/g. The present findings concluded that the test specimen G. *fergusonii* J. Ag commercially important than higher plants because of the availability and bioactivity.

Key words: Macro alga, *Gracilaria fergusonii*, Methanol, Spectral studies, Bioactivity

Introduction

Phenolic compounds are large group of natural products that are characterized by having at least one aromatic ring with one or more hydroxyl group attached. Phenolic compounds are considered to contribute to the health benefits associated to dietary consumption of fruits and vegetables. Recent advances of the analytical techniques have allowed some progress in their structural characterization. Seaweeds constituting an important marine living renewable resource occur generally on the rocky substratum in the intertidal and sub tidal regions of the coastal waters. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects reported by Benavente *et al.*, 1997; Manach *et al.*, 2005; Middleton *et al.*, 2000. The aim of the present study is confirmation of bioactive substance phenol and their anti-bacterial activity from the methanol extract of marine seaweed *Gracilaria fergusonii.*

Materials and methods

Collection and preparation of seaweeds

Marine red algae *Gracilaria fergusonii* J. Agardh were collected from Manapad coast of Tamil Nadu, India (8.3775°N; 78.0522°E) at low tide. Specimen was washed thoroughly in seawater to remove extraneous matter such as epiphytes and sand. After collection, fresh samples were taken into plastic jar and brought back to the laboratory immediately. Samples were washed by tape water for several times, then gently brushed and rinsed with distilled water and then dried at room temperature. The dried material was pulverized using domestic blender and extracted with organic solvent methanol by using soxhlet method. Finally, the extract of *Gracilaria fergusonii* stored for further uses.

FTIR Spectroscopic analysis:

FTIR spectrum was taken for the dried finely powdered sample about 1mg was mixed with about 100 mg of dried potassium bromide (IR grade) powder using SHIMADZU Perkin Elmer Spectrophotometer instrument. This was used to detect the characteristic peaks in ranging from 400-4000 cm^{-1}.

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UV –VIS Spectrophotometric Analysis:
The methanol extract of *G. fergusonii* was examined under UV-Visible spectral analysis. The extract was centrifuged at 3000 rpm for 10 min and filtered through Whatmann No. 1 filter paper by using high pressure vacuum pump. The sample is diluted to 1:10 with the same solvent. The extracts were scanned in the wavelength ranging from 200-1000 nm using 2371 Spectrophotometer and the characteristic peaks were detected.

NMR
2 to 40 mg purified compound of methanol extract of *G. fergusonii* was examined by ^{1}H NMR spectra dissolved in $CDCl_3$ were recorded at Bruker 300 MHz spectrometer equipped with a 5 mm probe. Chemical shifts were expressed as part per million (ppm) ^{1}H NMR used to determine the purified compound.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis
The dried seaweed powder sample was extracted with methanol using soxhlet extractor. The extract which is obtained in concentrated with rotary evaporator till dry powder was obtained. The final concentrated extract analyses by using GC-MS.
GC–MS analysis was carried out on equipment Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II. Experimental conditions of GC-MS system were as follows: ZB 5-MS capillary standard non-polar column, dimension: 30Mts, ID: 0.25 mm, Film thickness: 0.25µm. Flow rate of mobile phase (carrier gas: He) was set at 1.0 ml/min. In the gas chromatography part, temperature programme (oven temperature) was 70°C raised to 260°C at 6°C/min and injection volume was 1 µl. A sample dissolved in methanol was run fully at a range of 50-650 m/z and the results were compared by using Wiley Spectral library search programme.

Identification of Components
Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Antibacterial activity
Preparation of extract
5 gram of fine *G. fergusonii* species powder was dissolved in methanol. It was kept for 48 hours at room temperature, after 48 hours the solvent was filtered using whatman filter paper for further antibacterial assay.

Test Microorganisms Used
For anti-bacterial activity, human pathogens like gram positive bacterial strain *Staphylococcus aureus* and gram negative bacterial strains such as *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi* were used. The pure culture of the test pathogens were purchased from Proalgen Biotech Limited, Chengalpattu, Tamil Nadu and were maintained by culture in the laboratory condition.

Preparation of Inoculum
From the 24 hours incubated nutrient agar slant of each test organism a loop full of the bacterial strain was inoculated in nutrient broth at *p*H-7.4 so as to activate the bacterial strains used as test organisms. The broths were kept for incubation at 37°C for 24 hours so that the test organism can grow till the log phase. A nutrient broth was maintained as a control without inoculating the test organisms (Alvarez Benito, 1990).

Anti-bacterial Activity Test
Antibacterial activity was assayed using the agar well disc diffusion technique. Muller Hinton Agar Medium (MHA) was prepared; the *p*H is maintained at 7.4 and then sterilized by autoclaving at 121ºC and 15 lbs pressure for 15 minutes. 20 ml of the sterilized media was poured into sterilized petri dish and allowed to solidify at room temperature. A sterile cotton swab is used for spreading the test organism from the 24 hours inoculated broth evenly on the MHA plates. Similarly swabbing was done separately for each test microorganism on the MHA plates and left for few minutes to allow complete absorption of the inoculums. In each of these plates 5 mm diameter wells were made at the centre using an appropriate size sterilized cork borer.
Test extract was added to the respective wells on the MHA plates and allowed to diffuse at room temperature for 30 minutes. No extract was added in the control MHA plate which is used for comparing the obtained result from any contamination. The extract loaded plates were kept for incubation at 37°C for 24 hours. After incubation, a clear zone was observed around the well which was evidence of the presence of anti-bacterial active compounds in the test extracts. Diameters of the zone of inhibition were measured in millimeters (including the diameter of the well).

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Preparation of standard

For comparing the anti-bacterial activity of test extracts with the therapeutic action of a number of known broad spectrum antibiotics by Antibiotic Disc Diffusion Test was done. Ampicillin- AMP10-10 mcg/disc, Cefpodoxime- CPD10-10 mcg/disc, Erythromycin- E15- 15mcg/disc and Penicillin- P10-10 mcg/disc were used as standard. MHA was prepared and sterilized, after sterilization 20 ml of the media was poured into the sterile Petri dishes and allowed to solidify at room temperature.

Using sterile cotton swabs the test microorganism from the liquid 24 hours inoculated nutrient broth was spread evenly on each MHA plate. Using a sterile forceps each of the antibiotic disc of 10 µ mg was on the MHA plates and then kept for incubation at 37°C for 24 hours. Control MHA plates without any test microorganism were maintained. The diameter of the zone of inhibition was measured in millimeters. The zone exhibited by the test extracts was compared to the inhibition zones produced by the standard antibiotics. Standardize values of diameters of the inhibition halo, expressed in mm, produced by the microorganism against known antibiotics are listed in the literature by (Alvarez Benito, 1990).

Total free phenols

Quantification of total free phenols using Folin-Ciocalteu's method by Sadasivam and Manickam (1992). 500 mg of dried powdered sample was taken in a 100 ml flask to which 50 ml of 1% (v/v) HCl in methanol was added. The sample was shaken on a reciprocating shaker for 24 hours at room temperature. The content was centrifuged at 10000xg for 5 minutes. The supernatant was collected separately and used for further analysis. 1 ml of aliquot of the above extract was pipetted into different test tubes to which 1 ml of Folin Ciocalteu's reagent followed by 2 ml of 20% (w/v) Na_2CO_3 solutions were added and the tubes were shaken and placed in a boiling water bath for exactly 1 minute. The test tubes were cooled under running tap water. The resulting blue solution was diluted to 25 ml of distilled water and the absorbance was measured at 650 nm with using UV-Vis Spectrophotometer.

Results and Discussion

FTIR

Fourier Transform Infrared Spectrophotometer (FTIR) is the most powerful tool for identifying the functional groups present in the sample extract. From the FTIR results of *G. fergusonii* showed several absorbance peaks ranging from 560 cm^{-1} to 3500 cm^{-1} (Fig. 1). The functional phenol was observed the sharp peak showed range between 3300-3500 cm^{-1}. The FTIR spectrum was used to identify the functional group of the active compounds based on the peak value in the region of infra-red radiation. Those peaks from its analysis through IR chart confirmed the presence of the following functional groups in a compound. All the peaks with its respective functional group were illustrated in Table.1.

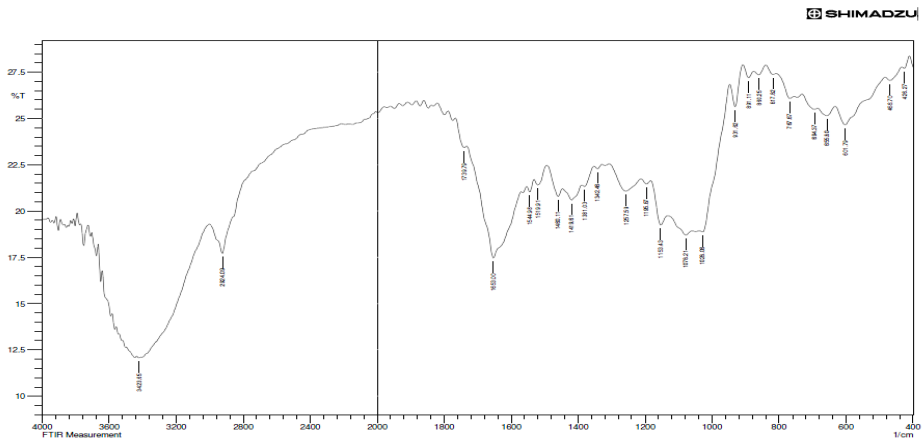

Figure 1: FT-IR spectrum of methanolic extract of *G. fergusonii*

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Table 1: Identification of functional groups of methanolic extract of *G. fergusonii* under FT-IR spectrum

No	Group frequency cm^{-1} of the sample compounds	Functional group compound	Functional Group assignment	Group frequency cm^{-1}
1	3423.65	Hydroxyl (Phenol, Alcohols)	OH stretching	3500-3300
2	2924.09	Methylene groups Alkanes	Asym.stretching	2925±10
3	1739.79	Aliphatic esters	C=O stretching	1740±5
4	1653.00	Amide	C=O stretching	1680-1630
5	1544.98, 1519.91	Nitroso compound	NO stretching	1600-1500
6	1460.11	Alkanes (Methyl group)	Asym. bending	1465±20
7	1419.61	Methyl group	Asym. bending	1420-1410
8	1381.03	Iso propyl	---	1385-1380
9	1342.46	Sulphur compound	SO_2 asym. stretching	1370-1330
10	1257.59	Phosphorus compound	P=O stretching	1300-1250
11	1195.87, 1153.43, 1078.21	Aliphatic esters	C-O-C asym. And sym. stretching	1300-1050
12	1028.06	Aryl and Vinyl ethers	C-O-C asym. stretching	1075-1020
13	931.62	Ethers	C-O-C asym.	950-810
14	891.11, 860.25, 817.82	Ethers	C-O-C asym. Stretching	950-810
15	767.67	Nitrite Ester	O-N stretching	815-750
16	694.37	Sulphur	S-O stretching	700-650
17	655.80	Sulphur (Sulphonic Acid)	S-O stretching	700-650
18	601.79	Nitrite ester	O-N=O bending	690-560

UV-Vis Spectral analysis

The UV-Vis spectral profile (Fig. 2) of the methanolic extract of *G. fergusonii* was observed at a wave length ranging from 200 to 1000 nm. The highest band was recorded at 315 nm with absorbance values of 2.459. The result of UV-VIS spectroscopic analysis confirms the presence of phenol in the methanolic extract of *G. fergusonii*.

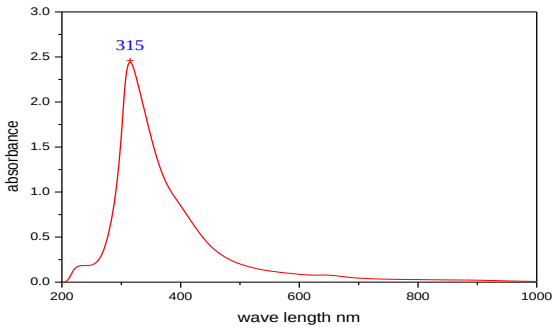

Figure 2 : UV-Vis spectrum of methanol extract *Gracilaria fergusonii*

NMR

The purified compound was subjected to ^1H NMR analysis that determined the number of hydrogen atoms present in the compound. The area under the plots provides information about the number of protons present in the molecule, the position of the signals (the chemical shift) reveals information regarding the chemical and electronic environment of the protons, and the splitting pattern provides information about the number of neighboring (vicinal or geminal) protons reported by Farlane, 1972. The ^1H NMR spectrum data showed Figure 3 and types of the proton and types of the compounds are present in Table 2. The H^1NMR the chemical shift ranges δ5.14ppm, δ 7.27 ppm, δ 7.46 ppm and δ 7.73 ppm were confirm the phenol group of tested methanol extract of *G. fergusonii*.

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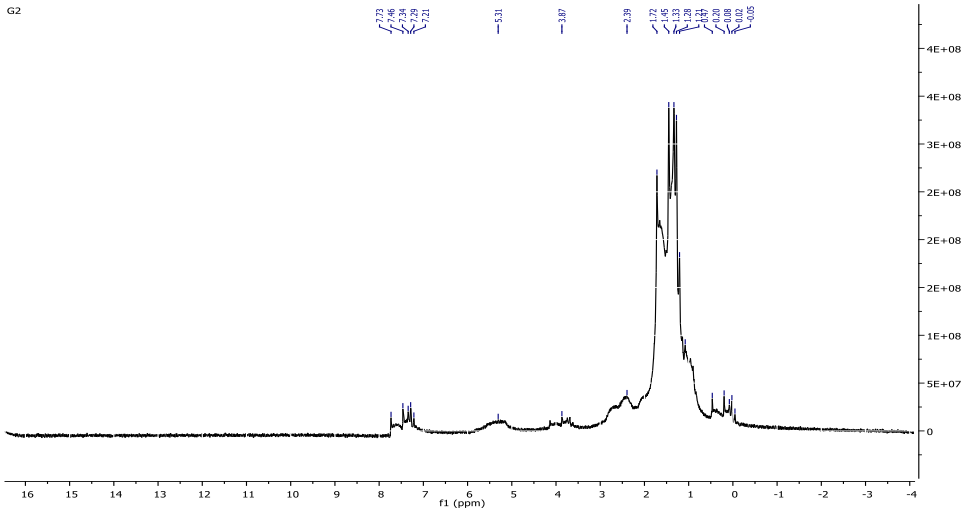

Figure 3: ^{1}H NMR spectra of methanol extract of *G. fergusonii*

Table 2: Confirmation of compound type ^{1}H NMR spectra of *G. fergusonii*

^{1}H NMR $CDCl_3$ ppm	type of proton	type of compound	chemical shift range, ppm
0.88	RCH_3	Alkyl (1° aliphatic)	0.8-1
1.25	RCH_2CH_3	Alkyl	1.2-1.4
1.61	R_3CH	Alkyl	1.4-1.7
2.28	$RCOCH_3$	Benzylic	2.1-2.3
2.33	$RCOCH_3$	ketone	2.1-2.6
3.67	$HOCH_2R$	Alcohol	3.3-4.0
5.14	ArOH	Phenolic	4.5-7.7
5.35	$R_2C=CHR$	vinylic	5.0-5.7
7.27, 7.46, 7.73	ArH	Aromatic	6.5-8.5

GC-MS Analysis

GC-MS analysis leads to the prediction of chemical constituent present in the methanol extract of *Gracilaria fergusonii.* The identification of the phytochemical compound was confirmed based on the peak area (%), Retention time (RT), molecular formula and molecular weight. GC-MS analysis of the phytochemicals present in methanol extracts of *Gracilaria fergusonii* clearly showed the presence of the compound Phenol, 2,4-Bis(1,1-Dimethylethyl)- is a phenolic compound with the RT 13.18 min, molecular formula $C_{12}H_{24}O$, molecular weight 206 and peak area 0.73% was showed in Figure 4 and 5. Phenolic compounds in particular are considered as one of the most important classes of natural antioxidants. Their molecules are formed by one or more aromatic rings with one or more hydroxyl groups.

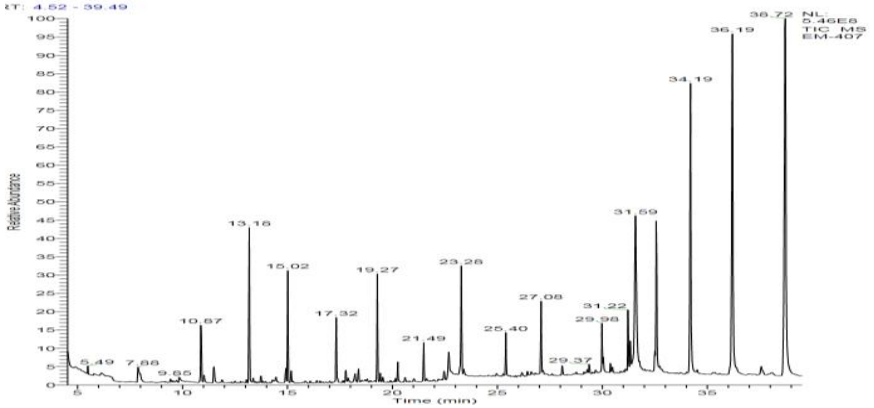

Figure 4: GC-MS chromatogram of chloroform extract of *Gracilaria fergusonii*

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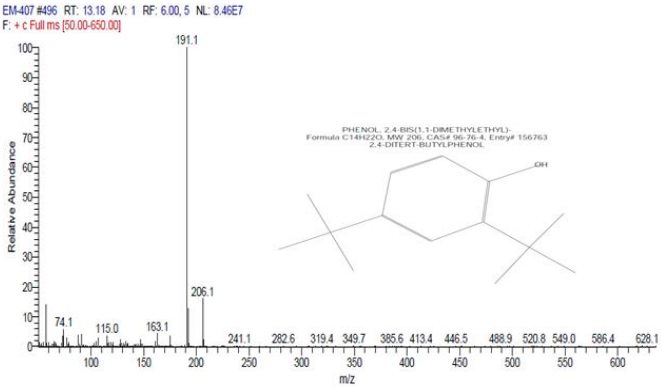

Figure 5: Mass spectrum and structure of Phenol, 2,4-Bis(1,1-Dimethylethyl)- (RT: 13.18)

Quantification of Total free phenol

The secondary metabolites like total free phenols were recorded in 3.08±0.022 mg/g. The secondary metabolites of seaweeds have always attracted the interest of biochemists because of their diversity as compared with those present in the leaves of higher plants. Phenols or polyphenols are compound containing a hydroxyl group attached an aromatic ring. It is readily soluble in organic solvents used as anesthetic and ointments. Flavonoids and coumarin are phenolic natural products occur in higher plants are used as health protection and pharmacological utility.

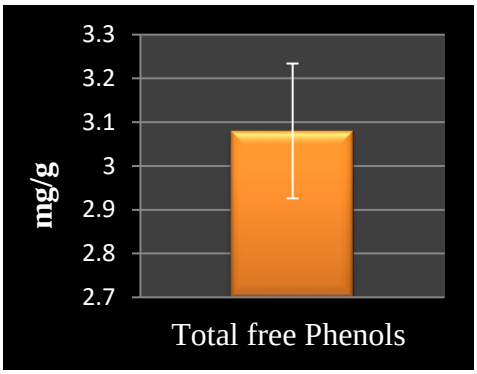

Fig 6: Quantification of Total Free Phenols of *G. fergusonii*

Antibacterial activity

In this assay, methanol extracts of *G. fergusonii* showed different anti-bacterial activity against human pathogenic bacterial strains such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococcus aureus.* The zone of inhibition of the anti-bacterial activity was observed by *G. fergusonii* extract against four bacterial human pathogen and where compared with the zone of inhibition of different antibiotics are presented in Table 3. Maximum zone of inhibition was observed at 3.5 mm against Gram negative *Pseudomonas aeruginosa*. Marine macro algae are eukaryotic organisms that live in salty water in the ocean and are recognized as a potential source of bioactive natural products (Micheal *et al.*, 2005). This supports the result obtained in this present study and proved that the marine algae contains biological active compound which is effective in resisting the growth of the human pathogenic bacteria.

Table 3: Anti-bacterial activity of methanol extract of *Gracilaria fergusonii* against human pathogens and standard antibiotics disc diffusion test in MHA media

Name of the microorganism	Gram^{+ve}/ Gram $^{-ve}$	Minimum inhibition concentration (MIC) Zone in mm	Name of the antibiotic discs used (mcg/disc)			
		Methanol extract of *G. fergusonii*	Ampicillin AMP10	Cefpodoxime CPD10	Erythromycin E15	PenicillinP10
E. Coli	-	-	-	-	-	-
Pseudomonas aurogenosa	-	3.5mm	3.5mm	-	-	-
Salmonella typhi	-	0.5mm	4mm	4.5mm	-	-
Staphylococcus aureus	+	-	-	-	-	-

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Conclusion

Algae are a source of many biologically functional substances including phenolic compounds which deserve attention because of the many health benefits they provide. The present findings concluded that the organic phenolic compound was confirmed in the algal specimen with the help of spectral techniques. The *G. fergusonii* was commercially important than higher plants because of the availability and bioactivity.

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