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FTIR, SEM, EDS and GCMS Metabolite Profiling of Macroalgae – *Sargassum wightii*

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ABSTRACT - *An extensive spectroscopic characterization of Sargassum wightii was performed to establish its complete metabolite profile. The samples were collected from Vedalai, Gulf of Mannar, Rameswaram, Tamil Nadu. The collected samples were processed and the SEM micrographs for cross sectional area of the lateral branch of the specimen was acquired. EDS analysis aided in the elemental analysis of the seaweed extract. FTIR technique was conducted to identify the frequency of functional groups in the S.wightii extract. The bands at 3408 cm^{-1}, 2926 cm^{-1}, 1643 cm^{-1} and 1423 cm^{-1} were indicating the presence of N-H, CH_3 and CH_2, O-H, C-H, C=O Stretching, N=O and C-O stretching vibrations in different compounds such as esters, amino acids, polysaccharides, starch, and carbohydrates respectively. Further, GCMS profiling aided in figuring out specific compounds like 8,10-Dodecadien-1-ol acetate and 18-oxo-methyl ester, 13-Docosenoic acid methyl ester which are of great pharmaceutical significance.*

KEY WORDS: *Sargassum wightii*, FTIR, SEM, EDS, GCMS

1.INTRODUCTION

Globally it has been reported that chlorophyll present in algae is the highest known source of chlorophyll. This green pigment is reported to be vital for rapid assimilation of amino acids. Aranzazu *et al.,* (2009) reported that red and green seaweeds contain carotenoids such as beta carotene, lutein, violaxanthin and fucoxanthin in brown seaweeds [1,8]. Yan *et al.,* (1999) found that the main carotenoids present in the red algae are beta-carotene, alpha carotene and their dihydroxylated derivatives such as zeaxanthin and lutein [22]. Nakamura *et al.,* (1996) revealed that algal polyphenol is called phlorotannin [16]. Phloratannin ranges from five to 15 % of dry weight in seaweeds. It plays an essential role in preventing disease linked to oxidative stress [7].

There are numerous reports on compounds derived from macroalgae with a broad range of biological activities, such as anti-bacterial [17], anti-viral, anti-tumor and anti-coagulant activity [3,13,23].

Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and cell damage. Free radicals are formed in both physiological and pathological conditions in different tissues [15]. The uncontrolled production of free radicals is considered as an important factor in the tissue damage induced by several pathophysiology's [19]. Anti-oxidants are compounds that dispose, scavenge and suppress the formation of free radicals or oppose them [14].

Yvonne *et al*., (2006) described that the Laminaria and Porphyra species has the potential to reduce the risk of intestinal or mammary cancer in animal studies [24]. Soo-Jin *et al.,* (2003) reported the potential anti-oxidative activities of enzymatic extracts from seven species of brown seaweeds [21]. The enzymatic extracts exhibited more prominent effects in hydrogen peroxide scavenging activity (approximately 90 %) and their activity was even higher than that of the commercial anti-oxidants.

Algae species have been shown to have bactericidal or bacteriostatic substances [9,20]. The anti-bacterial agents found in the algae include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, halogenated ketones, alkanes, cyclic polysulphides and fatty acids. In large number of marine algae anti-microbial activities are attributed to the presence of acrylic acid, phlorotannins, terpenoids and steroids.

Sargassum wightii, a dark-brown macroalgae, 20-30 cm in height with a well marked holdfast, upper portion richly branched, axes cylindrical, glabrous, leaves 5-8 cm long and 2-9 mm broad, leaves tapering at the base and apex, midrib inconspicuous vesicles large, spherical or ellipsoidal being 5-8 mm long and 3-4 mm broad, stipe of the vesicle 5-7 mm long seldom ending into a long tip, receptacles in clusters.

Sargassum wightii is widely employed in the production of alginate which chain are forming heteropolysaccharides made up of blocks of mannuronic acid and guluronic acid. It contains 8-10% mannitol which can be a substitute for

sugar, food and medicine. Fucose-containing sulfated polysaccharides are the main bioactive compounds responsible for the anti-tumor property of the algae [6].

Table -1: Scientific Classification of *S.wightii*

Domain	Eukaryota
Phylum	Heterokonta
Class	Phaeophyceae
Order	Fucales
Family	Sargassaceae
Genus	Sargassum

Kaliaperumal and Kalimuthu (1976) studied the seasonal changes in growth, reproduction and the content of alginic acid and mannitol in *Turbinaria deccurens* from Rameswaram coast [11]. Chennubhotla *et al*., (1982) found that alginic acid yield varies with the seasonal growth behaviour of *Sargassum ilicifolium* and *S. myriocystum* showing maximum yield in July to August and recommended the suitable harvesting period for getting the maximum yield of alginic acid between July and September [5]. Variation in growth and manitol content in *Padina gymnospora* conducted during 1975-1976 was reported by Chennubhotla *et al.,* (1977) [4]. Studies were made from September 1985 to August 1986 on the standing crop, algin and mannitol contents of three brown algae, *Colpomenia sinuosa, Hydroclathrus clathratus* and *Rosenvingea intricata* growing at Shingle Island and Kilakarai near Mandapam and there was no marked seasonal variation in the yield of algin and mannitol in these algae [12].

Seasonal variations in growth, alginic acid and mannitol contents of *Sargassum wightii* and *Turbinaria conoides* growing in the Gulf of Mannar near Mandapam were investigated for a period of two and a half years from August 1965 [18] and he observed that yield of alginic acid was high during the peak growth and fruiting periods, Mannitol content was at its maximum in the early stages of the growth cycle from May to August and minimum after the initiation of the reproductive receptacles.

Many have studied the yield and quality of sodium alginate on the pretreatment of *Sargassum wightii* with chemicals such as HCl, NaOH and formalin. Istini *et al*., (1994) compared the yield and physical properties of algin obtained from *Laminaria japonica, Eklonia cava* and *Sargassum duplicatum* collected from Japan [10]. Balakrishnan *et al.,* (2009) reported that among the alginophytes in the Gulf of Mannar area, *Stoechospermum marginatum* recorded the richest source of alginic acid closely followed by the species of Sargassum and Turbinaria [2].

Scanning electron microscopy (SEM) is a technique that uses electrons instead of light to form an output image. Since their development in the early 1950's, SEMs have thrown lights in many new areas of research including material science and nanotechnology. The SEM has allowed researchers to examine a much larger variety of specimens. The SEM has many advantages over traditional microscopes. The SEM has a large depth of field, which allows more of a specimen to be in focus at one time. The SEM also has much higher resolution; so closely spaced specimens can be magnified at much higher levels. Since SEM uses electromagnets rather than lenses, the researchers have much more control in the degree of magnification.

FTIR (Fourier Transform Infrared Spectroscopy) is a sensitive technique useful for identifying organic chemicals in a whole range of applications although it can also characterize some inorganic include paints, adhesives, resins, polymers, coatings and drugs. It is powerful tool for isolating and characterizing organic contamination. FTIR is the fact that the most molecules absorb light in the infra-red region of the electromagnetic spectrum. FTIR is particularly useful for identification of organic molecular groups and compounds due to the range of functional groups, side chains and cross-links involved which will have characteristic vibration frequencies in the infra-red range.

In the present study, the selected brown algae - *Sargassum wightii* sample is subjected to spectroscopic analysis employing SEM, EDS and FTIR to investigate its elemental concentration and functional groups. Further, the GCMS metabolite profiling of crude extract from *S.wightii* identifies its bioactive compounds defining its immunological properties.

2. MATERIALS AND METHODS

2.1 Collection of macroalgal sample

The brown seaweed *Sargassum wightii* was collected from Vedalai, near Rameswaram coastal region, Tamil Nadu. The *S.wightii* samples were cleaned from epiphytes, extraneous matter and necrotic were removed. Samples were collected in sterilized polyethylene bags, and transported to the laboratory. Samples were washed thoroughly with sea water then sterile distilled water, air dried, cut into small pieces and then ground until a fine powder is obtained.

Fig -1: Brown Macroalgae – *Sargassum wightii*

2.2 Scanning electron microscopy

A small piece of the lateral branch of *S.wightii* specimen was cut appropriately and washed with distilled water thrice without damaging the surface or clogging. The waxy outer layer was removed and the inner section was made into a thin cross-section fixed using glutaldehyde followed by freeze drying (critical point drying) placing it in a petri dish with filter paper beneath the specimen to ensure that structural integrity is maintained and no ice crystals are formed. The clean and dried sample was placed in the high vacuum environment using forceps with the sample stubs. The microphotographs were recorded using a scanning electron microscope (SEM JEOL model - JSE-5610LV, Japan) with an accelerating voltage of 20 kV, at high vacuum mode and Secondary Electron Image (SEI).

2.3 Energy Dispersive X-Ray Spectroscopy

Energy Dispersive X-Ray Spectroscopy (EDX) is a technique that provides the elemental curve as output. This analytical technique is generally used in conjunction with the SEM. EDX technique primarily detects the X-rays emitted from the sample during the process of bombardment by an electron beam for characterizing the elemental composition of the sample of interest. Quantitative results can be obtained from the relative x-ray counts at the characteristic energy levels for the sample constituents. Some typical applications include alloy identification, foreign material analysis, coating composition analysis etc. EDX helped to verify the presence of silver in the sample and its percentage as well. The semi quantification elemental analysis to identify the weight percentage of major and minor elements present in the samples was done using the OXFORD INCA Energy Dispersive X-ray Spectrometer (EDS).

2.4 Fourier Transform Infrared Spectroscopy

For FTIR, the *S.wightii* powder samples were prepared using the soxhlet apparatus using 70% ethanol as the organic solvent for extraction. 50g of the sample and 500ml of ethanol was taken for extraction and the apparatus was run for about 7 hours to get the concentrated extract of *S.wightii*. This final extract was further concentrated by evaporation and finally oven-dried to get a fine powder. 4 mg of the sample was mixed with 400 mg of FTIR grade potassium bromide and pressed into a pellet. The pellet was immediately placed in the sample holder and the infrared reflectance vibrational spectra was recorded in the range 4000-450 cm^{-1} with Perkin Elmer System One: FTIR at room temperature.

2.5 Gas Chromatography and Mass Spectrometry Analysis

The *S.wightii* extract was filtered on a Durapore-HV membrane filter disk with 2.5 cm diameter and 0.45 µm pore size by vacuum filtration. The filtrate was then transferred into a 1.5 ml eppendorf tube and frozen in liquid nitrogen. Frozen samples were stored at −80 °C till metabolite extraction. Metabolites were extracted immersing the filter in 1 ml of 90% (v/v) methanol containing 0.1 µg mL−1 U-13C-sorbitol followed by vortexing for about 5 seconds, the filter (attached to the eppendorf) was removed and the remaining solution was centrifuged at 20,000g for 5 minutes at 4°C. The sample was dried by a vacuum concentrator (SpeedVac concentrator, Thermo, Waltham, MA). Gas chromatography-mass spectrometry (GC-MS) analysis was performed by using JEOL GCMS system (JMS-GCMATE II, Japan). The GC column used was fused HyperSep silica capillary column (30 m X 0.25 mm X 0.25 µm) used with helium (carrier gas) at 1.51 mL for 1 minute. The mass spectrometer was operated in the electron impact mode at 70 eV. The split ratio was 1:10 and injection volume was 1 µL. The injector temperature was 250˚C while the set oven temperature was 70˚C/3 minutes, which rose to 250˚C/14 minutes. Mass start time was at 5 minutes and end time at 35 minutes. Peak identification of crude *Sargassum wightii* extract was performed by comparison with retention times of standards and the mass spectra obtained was compared with NIST libraries (NIST 11- Mass Spectral Library 2011 version) with an acceptance criterion of a match above a critical factor of 80%.

3. RESULT AND DISCUSSION

3.1 Scanning electron microscopy

The scanning electron microphotograph of the cross sectional area of the lateral branch of *S.wightii* was acquired at a magnification of 1000X. This SEM image can be used for measuring the effect of climate change on seaweeds or the cell wall changes in *S.wightii* due to salinity of seawater in different locations.

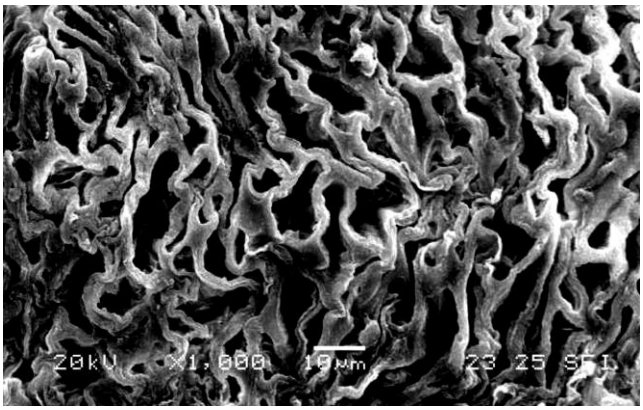

Fig -2: SEM micrograph of *S.wightii*

3.2 Energy Dispersive X-Ray Spectroscopy

The EDS spectrum depicts the x-rays of different macro and micro elements in the form of energy spectrum which in turn help in the identification of the concentration of elements such as sodium, magnesium, silicon, phosphorus, sulphur, chloride, potassium, calcium, manganese, iron and zinc thus aiding in creation of quantitative compositional profile for *S.wightii*.

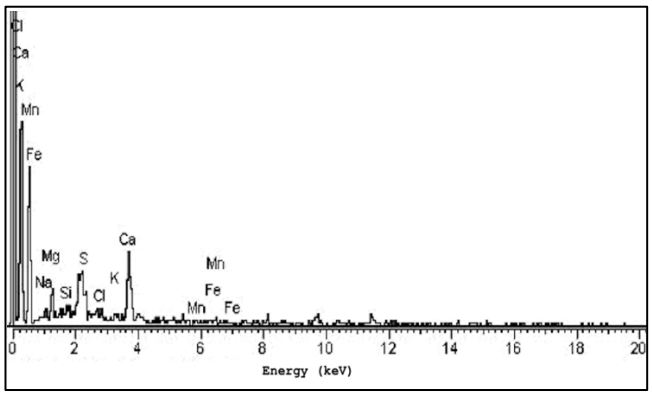

Fig -3: EDS spectrum of *S.wightii*

The EDS spectrum (figure 3; table 2) illustrates the high levels of chlorine, calcium, potassium, manganese and iron. The high level of chlorine is responsible for the osmotic regulation through ionic gradients of the seaweed in a high salinity environment while the calcium and potassium playing a significant role in the functioning of the cells and maintaining the water balance (Anderson et al., 2008). The manganese helps promote the protein metabolism nurturing the cell growth and development. Iron plays a vital role being a constituent of several enzymes and pigments helping the seaweed cope up with nitrate and sulfate reduction mechanisms.

These elements are known to be present as readily available form which is the reason behind the success of seaweed nutraceuticals/supplements and fresh seaweeds for human consumption.

Table -2: EDS elemental analysis of *S.wightii*

Characteristic Elements	Elemental Concentration (%)
Chloride	19.29
Calcium	3.72
Potassium	2.27
Manganese	0.30
Iron	15.75
Sulphur	21.90
Magnesium	2.33
Sodium	2.96
Silicon	9.89

3.3 Fourier Transform Infrared Spectroscopy

The FTIR spectrum aids in the identification of the molecular components and their structures.

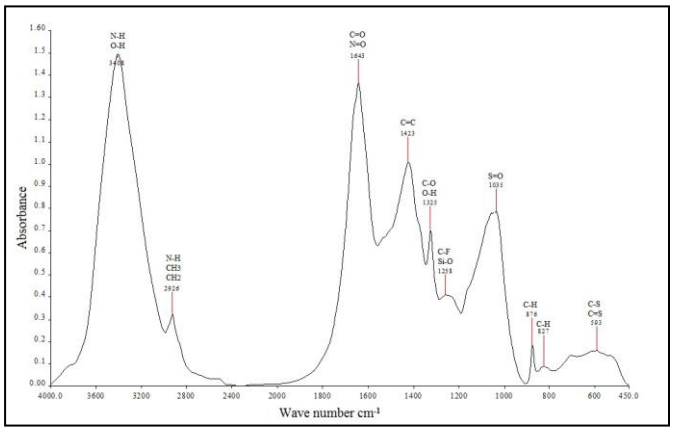

Fig -4: FTIR spectrum of *S.wightii*

The FTIR spectrum was used to identify the functional groups present in the solvent fractions of S. wightii (figure 4; table 3). The band appeared at 3408 cm^{-1} might be due to the strong N-H and O-H stretching vibrations corresponding to the amino acids and polysaccharides. The weak peak at 2926 cm^{-1} is attributed to the N-H Stretching and/or the CH3 and CH2 stretching vibrations of the aldehydes or saturated aliphatic groups.

A particular intense signal was recorded at 1643 cm^{-1} corresponding to the C=O Stretching and N=O asymmetric stretching of esters and pectin complexes. The absorption bands at frequencies 1423 cm^{-1}, 1258 cm^{-1} and 1035 cm^{-1} can be correlated with C=C stretching in lignin, C-F Stretching or Si-O bonding in cellulose or carbohydrates and S=O stretching indicating the presence of sulfonides in starch molecules or polysacchirides respectively.

Table -3: Functional group identification using FT-IR absorption frequencies (cm-1) for *S.wightii*

Absorption Frequency (cm^{-1})	Intensity Estimation	Functional Groups	Compound
3408	Strong	N-H Stretching O-H Stretching	Polysaccharides Amino acids
2926	Weak	N-H Stretching CH3 and CH2 stretching	Aliphatic Compounds
1643	Strong	C=O Stretching, N=O asymmetric Stretching (Nitrate)	Ester, Pectin
1423	Moderate	C=C Stretching	Lignin
1325	Weak	C-O Stretching O-H bending	Cutin
1258	Moderate	C-F Stretching Si-O	Cellulose, Carbohydrates
1035	Moderate	S=O Stretching (sulfonides)	Starch and polysaccharides
876,872	Weak	Out of plane C-H bending	Glucose, Galactose
593	Weak	C-S Stretching C=S Stretching (sulfides)	Sulfates

3.4 Gas Chromatography and Mass Spectrometry Analysis

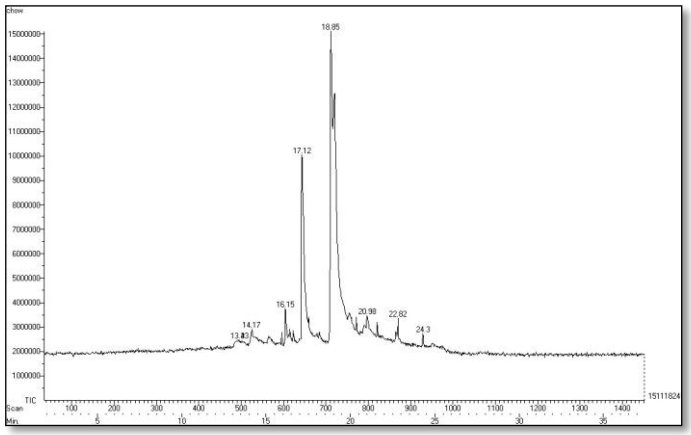

Fig -5: GCMS spectrum of *Sargassum wightii*

The compounds identified from *S.wightii* (brown algae) by interpreting the GCMS spectrum (figure 5) using the NIST library (table 4) were 8,10-Dodecadien-1-ol acetate, Cumarin-3-carboxylic acid-7-methoxy, Z-(13,14-Epoxy)-tetradec-11-en-1-ol acetate, Hexadecanoic acid methyl ester, 9-Octadecenoic acid (z) methyl ester, Nonadecanoic acid, 18-oxo-methyl ester, 13-Docosenoic acid methyl ester, Yohimbam-16-carboxylic acid, 17-hydroxy-methyl ester with their retention time, molecular weight and molecular formula.

Table -4: GCMS analysis and identification of compounds in *S.wightii*

Retention Time	Name of the Compound	Molecular formula	Molecular weight (g/mol)
13.43	8,10-Dodecadien-1-ol acetate	$C_{14}H_{24}O_2$	224
14.17	Cumarin-3-carboxylic acid-7-methoxy	$C_{10}H_6O_5$	220
16.15	Z-(13,14-Epoxy)-tetradec-11-en-1-ol acetate	$C_{16}H_{28}O_3$	268
17.12	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	270
18.85	9-Octadecenoic acid (z) methyl ester	$C_{19}H_{36}O_2$	296
21.02	Nonadecanoic acid, 18-oxo-, methyl ester	$C_{20}H_{38}O_3$	326
22.8	13-Docosenoic acid methyl ester	$C_{23}H_{44}O_2$	352
24.3	Yohimbam-16-carboxylic acid,17-hydroxy,methyl ester	$C_{21}H_{26}N_2O_3$	354

4. CONCLUSION

The Indian Ocean have abundant resources of brown seaweed *Sargassum wightii*, which have proven to have an innate effective defense system due to their adverse habitats. This study demonstrates the metabolite profiling and characterization of the bioactive compounds in *S.wightii* which can be further analyzed and isolated to be used in the production of pharmaceuticals and functional food supplements to treat several diseases such as hypertension, diabetes, and inflammatory disorders opening new frontiers in algal industry for this seaweed world-wide.

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