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## HPLC, FTIR AND GC-MS ANALYSIS OF ETHYL ACETATE EXTRACT OF RED SEAWEED *HYPNEA FLAGELLIFORMIS* GRAVILLE EX J. AGARDH 1851

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**ABSTRACT:** The experiment was carried out to investigate the phytochemicals constituents of the red seaweed *Hypnea flagelliformis* (Greville ex J. Agardh. 1851) from Tuticorin coastal waters, Gulf of Mannar, southeast coast of India; by using HPLC, FTIR, and Gas Chromatography-Mass Spectrometry. The selected seaweed was extracted with ethyl acetate (by soxhlet extraction method), furthermore subjected to HPLC, FTIR, and GC-MS analysis to reveal the phytochemical constituents. Totally five peak values were found in HPLC analysis. Among the five, three are a prominent peak at 2.243, 2.447, and 30.787 min, and the area percentages were 23.0, 45.4, and 30.6, respectively. The IR spectrum of ethyl acetate extract shows nine peaks corresponding to the wavenumber 3403.16, 2923.88, 2853.49, 2361.67, 1717.49, 1460.98, 1378.04, 1248.82, and 1042.45 cm<sup>-1</sup>. In the GC-MS analysis, a total of nineteen peaks were obtained, and the chromatograph showed 19 individual compounds. From all the nineteen compounds, three major compounds identified based on area percentage, which includes 1,4-eicosadiene (29.01%), n-tetracosanol-1(14.06%), and tetradecyl trichloroacetic acid (7.90%). Further, these novel bioactive structures can be optimized by using combinatorial chemistry generating new drug designs for future research.

**INTRODUCTION:** Plant-derived chemical compounds receive considerable attention because of their potential antioxidant, the antimicrobial and cytotoxic effect in the prevention and treatment of diseases. History reveals that plants are sources of successful drugs and will continuously be important for screening of new lead compounds<sup>1</sup>.

An essential part in the investigation of the plant through the identification of biologically active compounds present in plant leading to further biological and pharmacological studies<sup>2,3</sup>.

Currently, this technique is gaining popularity among different analytical techniques as an important and main choice for the study of fingerprinting for the quality control of herbal plants<sup>4</sup>. The biologically active entity is often present only as a minor component in the extract, and the resolving power of HPLC is ideally matched to the rapid processing of such multi-component samples on both an analytical as well as preparative scale<sup>5</sup>.

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The elucidation of its chemical structure can be addressed, and the characterization of the structure of bioactive compounds from the marine origin is also a challenging task. The structural elucidation is to ascertain what the skeleton of the molecule is, and this can often be narrowed down by references to literature on related genera and species. FT-IR is an important tool for measuring many chemical constituents present in plants<sup>6</sup> and seaweeds, and it is used to reveal some qualitative parts concerning the organic compounds. Several indicator bands that are pertained to functional groups represent chemical components or metabolic products. The application of infrared (IR) spectroscopy in plant analysis is still limited compared to its applications in other areas<sup>7</sup>. Further, the GC-MS method can be used for phytochemical profiling of plants and quantification of compounds present in plants, with increasing demand for herbal products as medicines and cosmetics there is an urgent need for standardization of plant products<sup>8</sup>. The optimized chromatographic fingerprint is not only an alternative analytical tool for authentication but also an approach to express the various patterns of chemical ingredients distributed in the herbal drugs and to preserve such a “database” for further multiracial sustainable studies. GC-MS fingerprint analysis has become the most potent tool for quality control of herbal medicines because of its simplicity and reliability. It can be used as a tool for identification, authentication, and quality control of herbal drug<sup>9</sup>.

In the present study, we evaluated the phytochemical constituents of ethyl acetate extract of *Hypnea flagelliformis* Greville ex J. Agardh 1851 belongs to Hypneaceae family by HPLC, FT-IR and Gas Chromatography and Mass Spectrometry (GC-MS), to provide the scientific information to generating new drug design for future research.

#### MATERIALS AND METHODS:

**Collection of Seaweed:** The seaweed *Hypnea flagelliformis* Graville ex J. Agardh 1851 was collected by Scuba diving at a depth of 3-5 meters, at Tuticorin coast of Gulf of Mannar during September 2014 to March 2015 (Lat 8°50'19.60835"N Long 78°15'22.19003"E). The taxonomic identification was done by the standard literature and the taxonomic keys<sup>10</sup>.

**Preparation of the Extracts:** Collected seaweed was transported to the laboratory in polyethylene bags filled with seawater. Seaweed sample was washed thoroughly with running water to remove epiphytes, animal castings, attached debris, and sand particles, and the final washings were done using distilled water and dried under shade. After that, the samples were cut into small pieces with the help of chopper and powdered in an electric blender. The seaweed powder was successively extracted using solvents of increasing polarity according to Arokiyaraj *et al.*, (2009)<sup>11</sup>, with slight modifications. Approximately 100 g of the powdered materials were extracted with 500 ml of ethyl acetate in a Soxhlet apparatus for 8 h at room temperature not exceeding the boiling point of the solvents. The extracts were filtered through Whatman no. 1 filter paper and then concentrated in vacuum at 40 °C by using a hot air oven. The residues obtained were stored in a freezer at -20 °C until further tests. The seaweed extract was further subjected to further study.

**HPLC Analysis:** The HPLC analysis of ethyl acetate extract of *H. flagelliformis* had been performed on a Shimadzu LC-10AT VP HPLC system, supported with a model LC-10AT pump, UV-VIS detector SPD-10AT, Rheodyne injector fitted with a 20 l loop and S908 an auto-injector SIL-10AT. A Hypersil BDS C-18 column (4.6 × 250 mm, 5 l m size) with a C-18 guard column was used. An isocratic HPLC (Shimadzu HPLC Class VP series) with two LC-10 AT VP pumps (Shimadzu), variable wavelength programmable photodiode array detector SPD-M10A VP (Shimadzu), CTO-10AS VP column oven (Shimadzu), SCL10A VP system controller (Shimadzu) and reverse phase Luna 5C18 (2) Phenomenex column (250 mm · 4.6 mm) was used.

The mobile phase components methanol: water (45:55) were filtered through 0.2 l membrane filter before use and were pumped from the solvent reservoir at a flow rate of 1 ml/min, which yielded a column backup pressure of 260–270 kgf/cm<sup>2</sup>. The column temperature was maintained at 27 °C. Twenty microliters of the respective sample were injected using a Rheodyne syringe (Model 7202, Hamilton). The elution was carried out with gradient solvent systems with a flow rate of 1 ml min<sup>-1</sup> at ambient temperature (25-28 °C). The

mobile phase was prepared daily, filtered through a 0.45  $\mu$ m, and sonicated before use. The sample injection volume was 20  $\mu$ l whilst the wavelength of the UV–VIS detector was set at 254 nm.

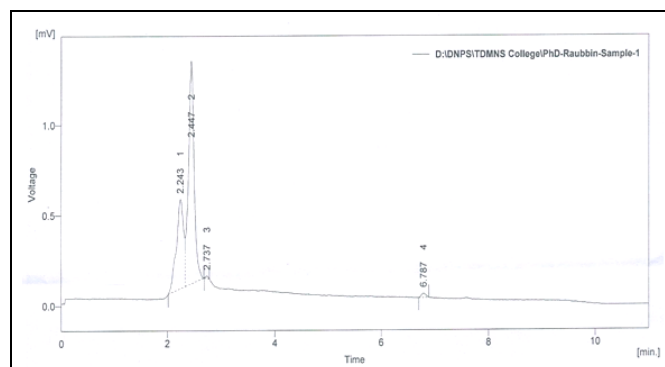
**FT-IR Analysis:** The functional groups of ethyl acetate extract of *H. flagelliformis* were identified by using an FT-IR instrument equipped with a Shimadzu spectrophotometer (made in Japan) and the peak values of FT-IR were recorded.

**GC-MS Analysis:** The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of ethyl acetate extract of *H. flagelliformis* was performed using an Agilent instrument, Gas Chromatograph connected to an Agilent 5977E mass selective detector equipped with an Agilent 7820A GC-auto sampler. The column used was a capillary column DB-5. Helium gas (99.9999%) was used as the carrier gas. GC-MS operating condition: column temperature, 10 °C hold for 1 min, at 80 °C 1 min to 280 °C; flow rate, 1.2 ml/min; injection port temperature, 280°C; injection volume, 1.5  $\mu$ l; injection mode, split, purge on after 1.5 min; ionization voltage, 70 eV; ion source temperature, 230 °C; GC-MS interface temperature, 280 °C; selected ion monitor mode: for each compound select one quantifying ion and two to three qualifying ions.

**Identification of Compounds:** The relative percentage of each extract constituents had been expressed as a percentage with peak area normalization. Interpretation was made by comparing their retention indices and patterns of mass spectra with reference to the National Institute of Standard Technology (NIST) library. The names, molecular weight, and structure of the components of the extracts were ascertained<sup>12</sup>.

## RESULTS:

**HPLC Analysis:** The HPLC fingerprint profile of *H. flagelliformis* extract showed five peak values in different retention times among the five, three are a prominent peak at 2.243, 2.447, and 30.787 min and the area percentage are 23.0, 45.4 and 30.6, respectively **Fig. 1** and **Table 1**.

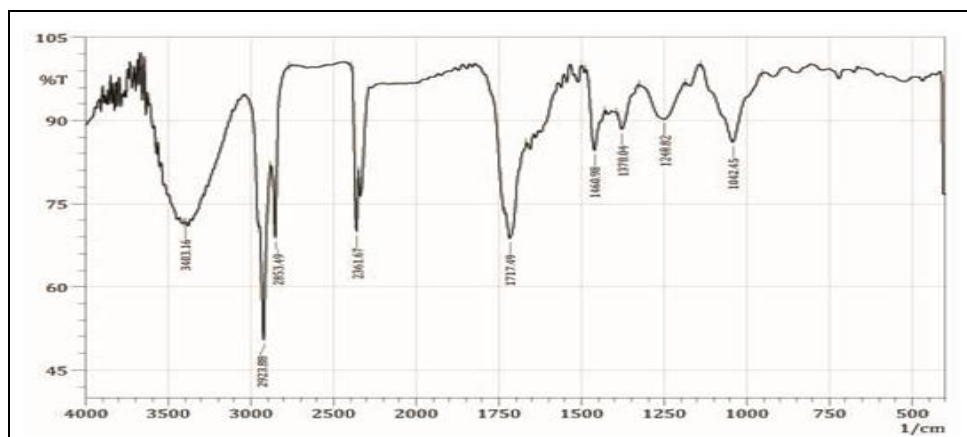


**FIG. 1: HPLC CHROMATOGRAM OF ETHYL ACETATE EXTRACT OF *H. FLAGELLIFORMIS***

**TABLE 1: HPLC ANALYSIS OF ETHYL ACETATE OF *H. FLAGELLIFORMIS***

Peak #	Reten. time	Area	Area%
1	2.243	4.603	23.0
2	2.447	9.080	45.4
3	2.737	0.050	0.3
4	6.787	0.137	0.7
5	30.787	6.145	30.6
Total		20.015	100

**FTIR Analysis:** The FT-IR spectrum was used to analyze the functional groups present in the ethyl acetate extracts of *H. flagelliformis*, and the results are revealed in **Table 2**. The IR spectrum of ethyl acetate extract shows several peaks corresponding to the wavenumber 3403.16, 2923.88, 2853.49, 2361.67, 1717.49, 1460.98, 1378.04, 1248.82, and 1042.45  $\text{cm}^{-1}$ .



**FIG. 2: FT-IR ANALYSIS OF ETHYL ACETATE EXTRACT OF *H. FLAGELLIFORMIS***

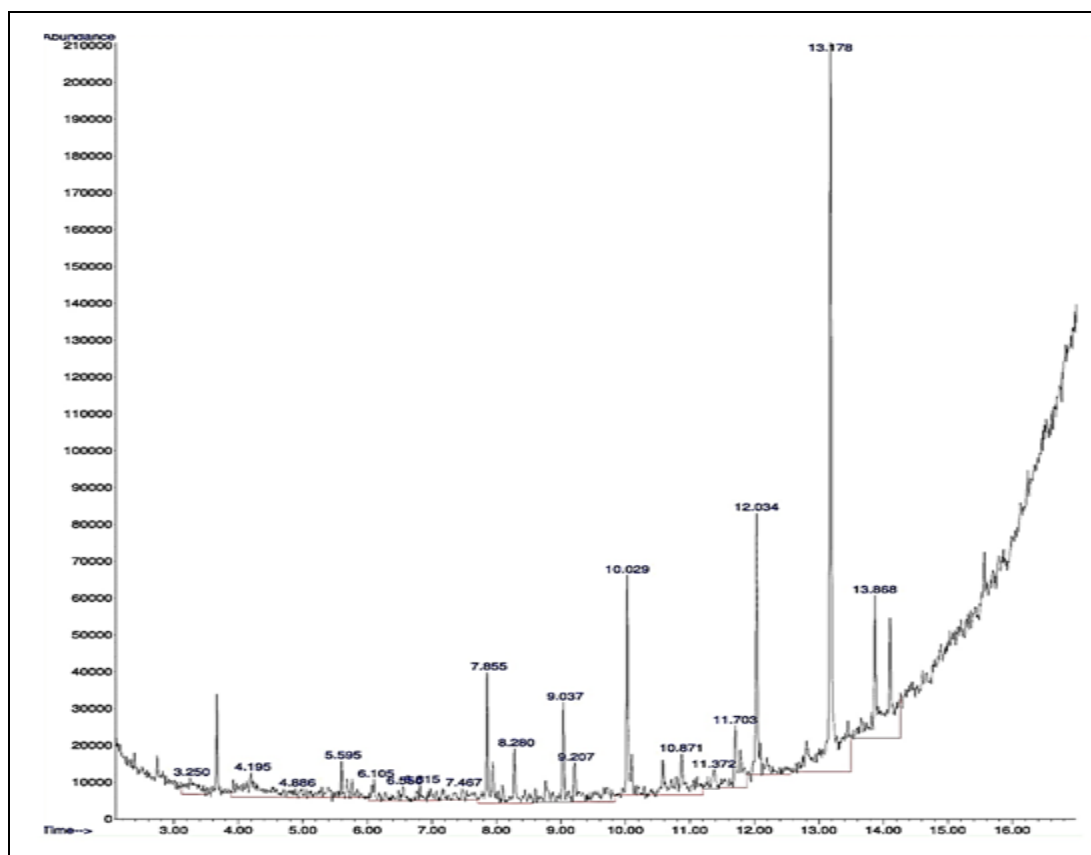
The peak at  $3403.16\text{ cm}^{-1}$  arises due to the O-H stretch of H-bonded alcohols and phenols. The peaks at  $2923.88$ ,  $2853.49$ , and  $2361.67\text{ cm}^{-1}$  are formed due to the O-H stretch of carboxylic acids, C-H stretch of alkanes, and the P-H phosphine respectively. The C=O stretch of carbonyls,  $\alpha$ ,  $\beta$ -unsaturated esters can be found at  $1717.49\text{ cm}^{-1}$ . The peak at  $1460.98\text{ cm}^{-1}$  attributes the C-C stretch of aromatics.  $1378.04\text{ cm}^{-1}$  represents the C-H stretch of alkanes. The C-N stretch of aromatic amines can be found at  $1248.82\text{ cm}^{-1}$ . The carboxylic acids, alcohols, esters, and ethers have C-O stretch at the peak of  $1042.45\text{ cm}^{-1}$  **Fig. 2**.

**TABLE 2: FUNCTIONAL GROUPS OF ETHYL ACETATE EXTRACT OF *H. FLAGELLIFORMIS***

S. no.	Peak Position	Corresponding Functional Groups
1	3403.16	-OH stretch, H-bonded alcohols and phenols
2	2923.88	-OH stretch of carboxylic acids
3	2853.49	-C-H stretch of alkanes
4	2361.67	P-H phosphine
5	1717.49	C=O stretch of carbonyls, $\alpha$ , $\beta$ -unsaturated esters
6	1460.98	C-C stretch of aromatics
7	1378.04	C-H stretch of alkanes (methyl)
8	1248.82	C-N stretch of aromatic amines
9	1042.45	C-O stretch of alcohols, carboxylic acids, ester and ether

**GC-MS Analysis:** GC-MS chromatogram for bioactive compounds of ethyl acetate extract of *H. flagelliformis* is shown in **Fig. 3**. On comparison of the mass spectra of the constituents with the NIST library, nineteen peaks were obtained which includes N-Allyl-N,N-dimethylamine (2.25%), 4-methyl-2-heptanone (4.91%), 2-hexyn-1-ol (1.23%), 1-tridecene (2.90%), 4-methyl-2-mercaptopyridine-1-oxide (1.30%), N-Isopropyl-3-phenylpropanamide (0.95%), tridecanal (1.40%), N-allyl-N,N-dimethylamine (1.75%), 1-nonadecene (4.31%), diphenylamine (2.93%), 1-hexadecane (4.05%), dodecanal (4.10%), 1-hexadecene (5.45%), 6,10-dimethyl-2-undecanone (5.94%), 10-undecenoic acid, 9-(3-oxooctanoyloxy)-, methyl ester (1.15%), n-hexadecanoic acid (4.39%), tetradecyl trichloroacetic acid (7.90%), 1,4-eicosadiene (29.01%) and n-tetracosanol-1 (14.06%).

From all the nineteen compounds, three major compounds identified, which includes 1,4-eicosadiene, n-tetracosanol-1, and tetradecyl trichloroacetic acid. The retention time, area percentage, compound name, molecular formula, and molecular weight are depicted in **Table 3**.



**FIG. 3: GC-MS CHROMATOGRAM OF ETHYL ACETATE EXTRACT OF *HYPNEA FLAGELLIFORMIS***

**TABLE 3: LIST OF COMPOUNDS IDENTIFIED FROM THE ETHYL ACETATE EXTRACT OF *H. FLAGELLIFORMIS***

S. no.	Rt	Area	Area (%)	Compound name	Molecular formula	Molecular weight
1	4.195	114039	4.91	4-Methyl-2-heptanone	C <sub>8</sub> H <sub>16</sub> O	128.212
2	7.855	100073	4.31	1-Nonadecene	C <sub>19</sub> H <sub>38</sub>	266.513
3	9.037	94087	4.05	1-Hexadecane	C <sub>16</sub> H <sub>34</sub>	226.448
4	9.207	95360	4.10	Dodecanal	C <sub>12</sub> H <sub>24</sub> O	184.323
5	10.029	126695	5.45	1-Hexadecene	C <sub>16</sub> H <sub>32</sub>	224.432
6	10.871	137968	5.94	6,10-Dimethyl-2-undecanone	C <sub>13</sub> H <sub>26</sub> O	198.345
7	11.703	102050	4.39	n-Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	257.422
8	12.034	183570	7.90	Tetradecyltrichloroacetic acid	C <sub>16</sub> H <sub>29</sub> Cl <sub>3</sub> O <sub>2</sub>	354.756
9	13.178	674164	29.01	1,4-Eicosadiene	C <sub>20</sub> H <sub>38</sub>	278.524
10	13.868	326793	14.06	n-Tetracosanol-1	C <sub>24</sub> H <sub>50</sub> O	354.663

**DISCUSSION:** The extracts of ethyl acetate were obtained from *H. flagelliformis* through selective sequential extraction with solvents of increasing polarity further, HPLC, and the functional group's data was provided by the FT-IR for ethyl acetate extract which showed typical absorption bands to know the functional groups. On the other hand, ethyl acetate extract's 'GC-MS analysis' revealed that nineteen bio-active compounds were present.

There were very less therapeutic applications, nor phytochemical studies about this seaweed are cited in the literature, and this represents the first study in Gulf of Mannar, Tuticorin coastal waters (at a depth of 3 – 5 meters) to elucidate the functional group and compounds analysis using GC-MS about this red seaweed *H. flagelliformis*.

In the present study, bioactive compounds were identified from the *H. flagelliformis*, and it was compared with the other seaweed or plant species compounds already recorded<sup>13, 14, 15</sup>. The modern analytical tools like HPTLC and HPLC confirmed the presence of the active constituents and potential adulterants in ayurvedic drugs<sup>16</sup>.

In this study, the HPLC fingerprint profile of ethyl acetate extracts of *H. flagelliformis* showed five sorts of compounds at different peak values. Johnson *et al.*,<sup>17</sup> studied *S. wightii* from Rasthacaud, Kanyakumari District, and found nine compounds in chloroform extracts and four compounds in benzene extracts. Issac *et al.*,<sup>18</sup> investigated the bioactive compounds of *H. musiformis* from Tuticorin coastal waters. The *H. musiformis* extracted by methanol elucidated the phenolic compounds of gallic acid, epigallocatechin gallate, catechin gallate, catechin hydrate, epigallocatechin, epicatechin, epicatechin gallate, pyrocatechol and 4-hydroxybenzoic acid.

The functional combinations of the dynamic components have been distinguished by the use of FT-IR spectrum analysis based on this outcome ethyl acetate extract of *H. flagelliformis* exhibited that nine functional groups were present in **Table 2**. Sumayya *et al.*,<sup>19</sup> analyzed (FT-IR spectroscopy) the functional group of *H. muciformis* (red seaweed) collected from Mandapam coast Gulf of Mannar and reported the peak values of 4000-900 cm<sup>-1</sup>, the functional groups are alcohols/phenols, alkanes, nitro groups, ketones, saturated aliphatic, aliphatic amines and carboxylic acids were reported.

Likewise, the FT-IR analysis of *Padina tetrastratica* and *Sargassum oligocystum* from Hare Island in Gulf of Mannar studied by Rani *et al.*,<sup>20</sup> and reported carboxyl, sulfhydryl and hydroxyl are the prime constituents, furthermore aliphatic compounds, starch, polysaccharides, and glucose peak values commonly present in both the seaweeds.

The GC-MS analysis of ethyl acetate extract displays the highest peak area percentage in 1,4-eicosadiene with 29.01%, and this compound was also present in some other species like *Streptocaulon sylvestre* (1.94%), *Paronychia kapela* (7.33%) and *Plectranthus amboinicus* (1.33%) and reported antioxidant activity<sup>21</sup>. Moreover, n-tetracosanol-1 (14.06%) was also reported as a major compound in ethyl acetate extracts of *H. flagelliformis* and they also present in plants like *Acrocarpus fraxinifolius* (5.46%), *Clinacanthus nutans* (1.72%), *Glycyrrhiza glabra* (1%), *Zea mays*, *Croton bonplandianum* (5.67%), *Paracoccus pantotrophus* (4.64%), *Terminalia travancorensis* (8.21%) and reported activities such as antimicrobial activity, anticancer, antioxidant activity, antimalarial, antiplasmodial potentials,

peroxidation, enhancing immune functions, inhibits platelet aggregation, cardiovascular, hepato-protective activity, antiproliferative effect<sup>22, 23, 24, 25, 26</sup>. Furthermore, the compound tetradecyl tri-chloroacetic acid was also observed in the same extract with the peak area percentage of 7.90%, but there was no report about this compound in any other plants. Based on the GC-MS analysis, some significant chemical constituents have been detected in the ethyl acetate extract of *H. flagelliformis* that can be additionally separated, purified as well as verified to be used for the purpose of medicines.

**CONCLUSION:** The knowledge gained through the present study paved the way to find out the unknown molecules to be identified in the seaweed *H. flagelliformis*. These novel bioactive structures can be optimized, and further pharmacological investigation on *H. flagelliformis* needed to explore the unknown compound's isolation and purification for generating new drug design.

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