

Biochemical Study and GC-MS Analysis of *Hypnea musciformis* (Wulf.) Lamouroux

Mohan Balamurugan, Govindarasu Ganapathy Selvam,
Thillaikkannu Thinakaran and Kathiresan Sivakumar

Division of Algal Biotechnology, Department of Botany, Faculty of Science,
Annamalai University, Annamalai Nagar-608602, Tamil Nadu, India

Abstract: *Hypnea musciformis* (red seaweed) was investigated for the richness of its bioactive compounds. The ethanolic extract was subjected to GC-MS analysis revealed 26 chemical constituents. Seaweed exhibits potentially bioactive major constituents like n-hexadecanoic acid-tetradecanoic acid, oleic acid-9-octadecenoic acid-6-octadecenoic acid, hexadecanoic acid-ethyl ester-ethyl tridecanoate and octadecanoic acid. Further biochemical and mineral analyses presented the phenolic content, total carbohydrate, total protein, fat, moisture content, ash, vitamins A, C and E and lipid soluble and water-soluble antioxidants. Chemical elements, such as Ca, Mg, Na, K and Fe were in highest proportion in the red seaweed.

Key words: *Hypnea musciformis* • Ethanolic extract • Gas chromatography • Bioactivity

INTRODUCTION

Seaweeds grow in the intertidal as well as in the subtidal area up to a certain depth where 0.1% photosynthetic light is available; they are one of the ecologically and economically important living resources of the world ocean. They are able to biosynthesize secondary metabolites that can mediate a broad range of intra and inter specific ecological interactions between marine organisms, including chemical defenses against herbivores [1, 2].

Marine algae are rich sources of structurally new and biologically active metabolites [3]. In recent years, there have been many reports of macroalgae derived compounds that have a broad range of biological activities, such as antibiotic, antiviral, antioxidant, antifouling, anti-inflammatory, cytotoxic and antimutagenic activities [4]. Seaweeds are known to contain reactive antioxidant molecules, such as ascorbate and glutathione (GSH) when fresh, as well as secondary metabolites, including carotenoids (α - and β -carotene, fucoxanthin, astaxanthin), mycosporine-like amino acids (mycosporine-glycine) and catechins (e.g., catechin, epigallocatechin),

gallate, phlorotannins (e.g., phloroglucinol), eckol and tocopherols (α -, x -, δ -tocopherols) [5]. Several studies have reported on the photochemistry of seaweeds during last decade [6]. The written record of the study of marine plants dates back to the third century B.C with the Greek naturalist Theophrastus, who gave descriptive account of certain copious and useful seaweeds.

Seaweeds concentrate minerals and trace elements from marine water and convert them in organic forms as they grow in a mineral-rich medium [7]. The numerous elements coming from the sea are Ca, Cl, Cu, I, Mg, Mn, Na, P, S and Zn [8]. They selectively absorb elements like Na, K, Ca, Mg, I and Br from the seawater and accumulate them in their thalli. The accumulated elements vary from species to species. For example, large quantities of K and I are taken up by many brown seaweeds and Ca and Br by red algae. Marine algae generally contain Na, K, Ca, Mg and Fe in large quantities, up to 15%–25% of dry weight. The inorganic content appears very high when compared with 5–6% in hay or nearly 4% in cereals. Seaweeds are known as alkaline food, as their inorganic components play a very important role in preventing blood acidosis [9].

Corresponding Author: Dr. K. Sivakumar, Division of Algal Biotechnology, Department of Botany,
Faculty of Science, Annamalai University, Annamalai Nagar-608602, Tamil Nadu, India.

Hypnea musciformis belongs to the family Hypneace, whose plants are bushy, spreading, cylindrical, 10-30 cm high, purplish green in color, cartilaginous, much branched, branches irregular, giving a bushy look to the plant [10]. The hooked and swollen tendrils are the characteristic feature of this species. It has been reported to possess k-carrageenan [11, 12]. Carrageenan is extensively used as a food additive in a wide range of products including cheese, cream, chocolate and ice creams. Its chief use is as a suspending and stabilizing agent and has a number of pharmacological properties [13]. The study was designed to investigate the phytochemicals potential in *H. musciformis*, biological activity and potential development of novel, environment-friendly formulations using GC-MS.

MATERIAL AND METHODS

Collection and Extraction of Seaweed: *H. musciformis* (red seaweed) was collected from the intertidal and subtidal habitat of the Pudumadam coastal area located on the southeast coast of Tamil Nadu, India. The collection was performed during the January 2013, when red algal diversity remains dominant. Live and healthy plants were harvested manually and washed thoroughly in running water to remove epiphytes, animal castings, sand and calcareous and other adhering detritus matters. Cleaned seaweed materials were shade dried under an air jet to prevent photolysis and thermal degradation. The completely dried material was weighed and ground coarsely in a mechanical grinder.

Biochemical Analysis

Determination of Lipid-soluble Antioxidant Capacity (CALT): Samples were homogenised with hexane and shaken for 1 h at 4°C in the dark. After centrifugation at 6000 g for 10 min, the supernatant was transferred to new tubes. Samples of ethanolic extracts (200 µL) were placed in Eppendorf tubes, dried out and re-dissolved in the same volume of ethanol. These ethanolic solutions were supplemented with 1 ml phosphomolybdenum reagent (32 mol/L. sodium phosphate, 4 mol/L. ammonium molybdate, 0.6 M sulfuric acid) and were incubated at 95°C for 90 min. Finally, the absorbance was measured at 695 nm. Lipid-soluble antioxidant capacity is expressed as equivalents of α -tocopherol [14].

Determination of Water-Soluble Antioxidant Capacity (Caht): Samples of water extracts (200 µL) were supplemented with 1 ml phosphomolybdenum reagent

and incubated at 95°C for 90 min. Finally, the absorbance at 695 nm was measured. Water-soluble antioxidant capacity is expressed as equivalents of L-ascorbic acid [14].

Total Phenolic Content: Phenolic contents of crude methanol extract were measured using Folin Ciocalteu's method as described by Taga *et al.* [15]. Absorbance was measured at 720 nm and total phenolic content was calculated with a gallic acid standard and expressed as gallic acid equivalent per gram.

Determination of Vitamin E: Vitamin E content was determined by using a method described by Prieto *et al.*, [14]; 0.1 ml hexanic extract of algae was mixed with 1 ml phosphomolybdenum reagent solution and incubated at 37°C for 90 min with vigorous shaking. The absorbance was measured at 695 nm. Vitamin E content was expressed as α -tocopherol equivalents per gram of extract.

Determination of Vitamin C: Ascorbic acid concentrations were determined by the titrimetric Association of Official Analytical Chemists (AOAC) method No. 967.21 using 2,6-dichlorophenol indophenol as a titrant [16].

Determination of Vitamin A: The samples were extracted with ethanol. Vitamin A content was determined and calculated by using a method described by Rutkowski *et al.* [17].

Total Protein: Total protein content was determined spectrophotometrically at 595 nm and concentrations were calculated by comparison to a calibration curve of bovine serum albumin [18].

Determination of Total Soluble Carbohydrate: Total soluble carbohydrate was assayed by the anthrone-sulfuric acid method [19], involving extraction with 15% trichloroacetic acid. The absorbance was measured at 620 nm.

Moisture Content and Ash Value: Moisture content was determined by complete drying of the sample at 100±5°C i.e., by complete removal of free water present in the sample. The loss in weight of the sample is the measure of moisture content [16]. The ash content of the sample is the inorganic residue left after complete removal of the organic residue by muffling at about 550-650°C in the muffle furnace according to the standard procedure of AOCS [16].

Minerals by Flame Atomic Absorption Spectrophotometry: Ashes were dissolved in nitric acid (analytical grade Merck, Germany) and passed through an ash-free, acid washed filter paper (Albert No. 242, 9 cm diameter). Major mineral elements (Na, K, Ca and Mg) and trace element (Fe) were determined in a Perkin Elmer 5100 PC atomic absorption spectrophotometer, equipped with single hollow cathode lamps for each element and an air-acetylene burner.

Preparation of Ethanolic Extract (*H. musciformis*) for GC-MS Analysis: *H. musciformis* was shade dried and 2 g of the powdered biomass was soaked in 95% ethanol for 12 hr. Then the extract was filtered through Whatman No 41 filter paper along with 0.2 g of sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper was moistened with 95% ethanol for 12 hr. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phyco-components of the seaweed material used. An aliquot of 2 μ l of this solution was employed for GC-MS analysis [20].

GC-MS Analysis: GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system comprising of a Aoc-20i auto sampler and gas chromatograph interfaced to a mass spectrometer. GC-MS instrument employed the following conditions: column-Elite-1 fused silica capillary column (30 mm x 0.25 mm ID x 1 μ Mdf), composed of 100% dimethyl poly siloxane, operating in electron impact mode at 70eV; carrier gas-helium (99.999%) at a constant flow of 1 ml/min; injection volume-0.5 μ l (split ratio of 10:1); injector temperature- 250°C and an ion source temperature of 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 0°C/min to 200°C, then 5°C/min to 280°C ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 min.

RESULTS

The determined bioactive compounds of *H. musciformis* was total carbohydrate, total protein, fat, moisture content, ash, vitamins A, C and E, total phenol, lipid-soluble antioxidant capacity (CALT) and water-soluble antioxidant capacity (CAHT). Amount of these compounds were shown in Table 1.

The total phenolic content of *H. musciformis* was 0.61 \pm 0.04mg gallic acid equivalent per gram dry seaweed. Furthermore, total carbohydrate, total protein, fat,

Table 1: Phycochemical analysis and Antioxidant capacity of *Hypnea musciformis* (Wulfen) Lamour.

S. No.	Phycochemicals and Antioxidant capacity	<i>Hypnea musciformis</i> (Wulfen) Lamour \pm SD
1.	Total Carbohydrate (mg/g)	55.82 \pm 3.72
2.	Total Protein (mg/g)	12.15 \pm 1.34
3.	Fat (mg/g)	0.61 \pm 0.08
4.	Moisture content (mg/g)	16.17 \pm 2.10
5.	Ash (mg/g)	11.72 \pm 1.16
6.	Vitamin - A (μ M)	1.25 \pm 0.47
7.	Vitamin - E (mg/g)	0.41 \pm 0.13
8.	Vitamin - C (mg/g)	0.72 \pm 0.12
9.	Total phenol (mg/g)	0.61 \pm 0.04
10	CALT (μ m α -Tocopherol/g)	251.13 \pm 87.52
11.	CAHT (μ m L-ascorbic acid/g)	991.54 \pm 77.23

SD = Standard Deviation; CALT = lipid-soluble antioxidant capacity; CAHT = water-soluble antioxidant capacity.

Table 2: Analysis of mineral content in *Hypnea musciformis* (Wulfen) Lamour.

S. No.	Mineral contents (mg/g)	<i>Hypnea musciformis</i> (Wulfen) Lamour \pm SD
1.	Calcium	10.18 \pm 1.52
2.	Magnesium	12.31 \pm 1.34
3.	Sodium	14.93 \pm 1.57
4.	Potassium	10.15 \pm 1.19
5.	Iron	1.62 \pm 0.09

SD = Standard Deviation

moisture content, ash, vitamins A, C and E, lipid soluble and water soluble antioxidant capacities were found to be 55.82 \pm 3.72 mg/g, 12.15 \pm 1.34 mg/g, 0.61 \pm 0.08 mg/g, 16.17 \pm 2.10mg/g, 11.72 \pm 1.16mg/g, 1.25 \pm 0.47 μ M, 0.41 \pm 0.13mg/g, 0.72 \pm 0.12 mg/g, 251.13 \pm 87.52 μ mol - tocopherol/g and 991.54 \pm 77.23 μ mol L-ascorbic acid/g, respectively.

All the minerals, such as calcium, magnesium, sodium, potassium and iron were found to be 10.18 \pm 1.52mg/g, 12.31 \pm 1.34 mg/g, 14.93 \pm 1.57 mg/g, 10.15 \pm 1.19 mg/g and 1.62 \pm 0.09 mg/g, respectively (Table 2) in *H. musciformis*.

GC-MS Profile of Ethanol Extract of *Hypnea musciformis*: A high resolution mass spectrum equipped with a data system in combination with Gas Chromatography was used for the chemical analysis of active red seaweed. The crude ethanolic extract of *H. musciformis* based on spectral data by GC-MS analysis was found to be a mixture of volatile compounds. 26 peaks were observed with retention times as presented in Fig. 1 and Table 3.

The GC-MS analysis of the crude extract revealed that the main phyco-constituent was n-Hexadecanoic acid- Tetradecanoic acid (RT 18.205, 57.89%) Oleic

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 Acquired : 16 May 2013 17:54 using AcqMethod UN_KNOWN.M
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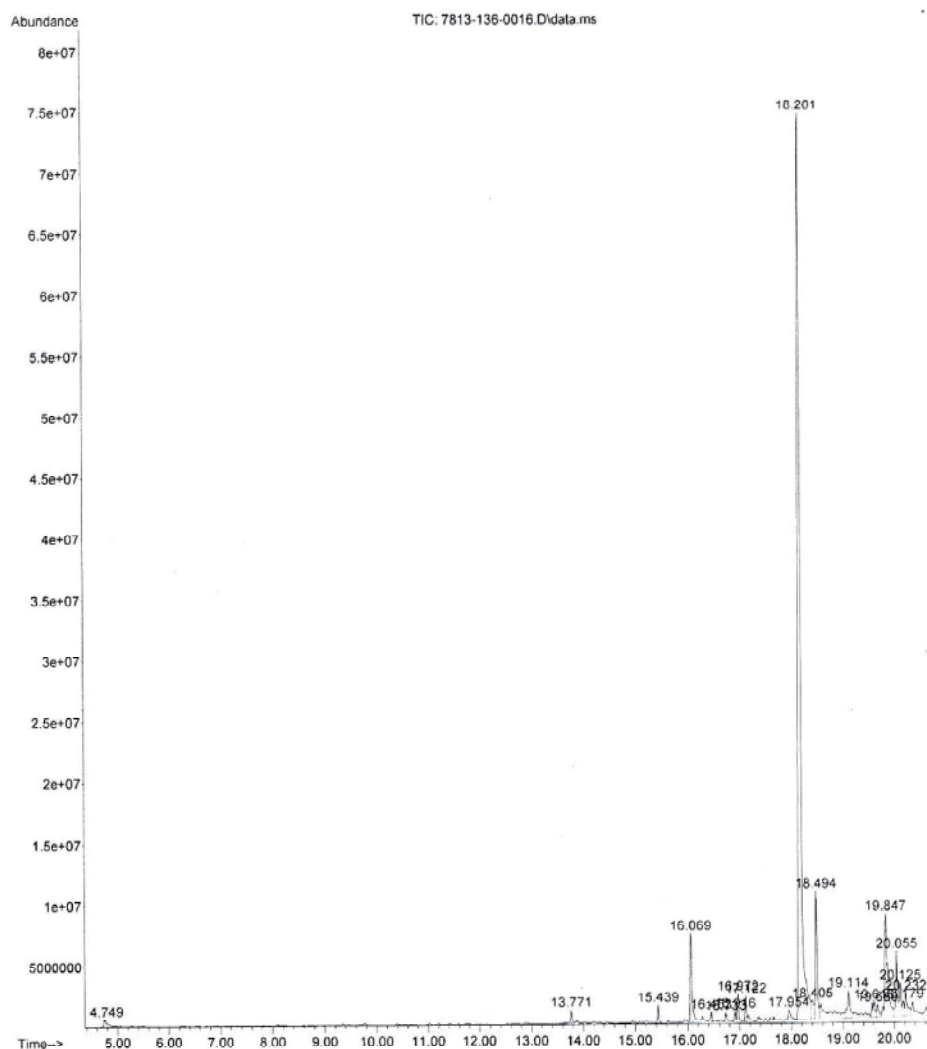


Fig. 1: GC-MS analysis of phytochemicals identified from ethanolic extract of *Hypnea musciformis* (Wulfen) Lamour.

acid-9-Octadecenoic acid-6-Octadecenoic acid (RT 19.846, 7.08%), Hexadecanoic acid-ethyl ester-Ethyl tridecanoate (RT 18.496, 5.16%) and Octadecanoic acid (RT 20.050, 2.77%) may be involved in biological activity. Seaweeds exhibit a high level potential bioactivity.

DISCUSSION

In our opinion, the antigenotoxic and chemo-protective effect may have originated from the anti-oxidative molecules of HME *in vitro*. Plants contain

different antioxidants and considering that vitamin E biosynthesis occurs only in plants, it would be useful to know the total concentration of antioxidants in individual species. Phenolic compounds and total antioxidant capacity have a major effect on antioxidative activities [21]. Therefore, a number of studies have focused on the biological activities of phenolic compounds. Several authors have reported increased antioxidant and antimutagenic/anticarcinogenic activities due to these compounds [22-24]. In the present study, *H. musciformis* was found to have rather higher total phenol

Table 3: GC-MS analysis of phytochemicals identified from ethanolic extract of *Hypnea musciformis* (Wulfen) Lamour.

S. No.	RT	Name of the compound	Peak area%
1.	4.743	Glycerine	0.72
2.	13.776	Dodecanoic acid	0.51
3.	15.446	Heptadecane	0.52
4.	16.070	Tetradecanoic acid	4.15
5.	16.448	Tetradecanoic acid ethyl ester	0.42
6.	16.739	Pentadecanoic acid, Oxirane, Tetradecyl	0.32
7.	16.913	Bicyclo (3,1,1) heptane, 2,6,6 trimethyl, phytol, acetate Bicyclo (3,1,1) heptane, 2,6,6 trimethyl, (1R - (1-alpha, 2-beta., 5-alpha))	0.31
8.	16.971	2-pentadecanone, 6,10,14, trimetyl	0.98
9.	17.116	Pentadecanoic acid	0.76
10.	17.958	Palmitoleic acid,cis-9-Hexadecenoic acid, Z-7-Hexadecenoic acid	0.58
11.	18.205	n-Hexadecanoic acid, Tetradecanoic acid	57.89
12.	18.409	n-Hexadecanoic acid, Tridecanoic acid	1.09
13.	18.496	Hexadecanoic acid, ethyl ester, Ethyl tridecanoate.	5.16
14.	19.120	1-Metoxo-9-methylphenazineDibenzo(d,f)-1,3,2-dioxaborepine, 2-ethyl-1-methyl-6-Methyl phenazine	2.24
15.	19.614	7-8-Hexadecane cyclohexadecane Heptafluorobutyric acid, pentadecyl ester.	1.07
16.	19.687	1,3-Diamino-7-methyl benzo(8) quinazoline. Pyrazole-5-methyl-1-phenyl-4(3-pyrazolyl)-S triazolo (1,5-a) pyrazine, 5,8-dimethyl-2-phenyl	0.65
17.	19.846	Oleic acid, 9-Octadecenoic acid, 6-Octadecenoic acid.	7.08
18.	20.050	Octadecanoic acid	2.77
19.	20.122	Ethyl oleate, Ethyl oleate 9-Octadecenoic acid, ethyl ester	1.30
20.	20.181	9-Octadecenoic acid, (E)-Ethyl Oleate. Ethyl oleate	0.59
21.	20.239	9-Octadecenamamide, (Z)-2-Methyl phenothiazine Tetra delanamide	1.46
22.	21.647	Cycloheptanol, 2-methylene, 1,1-Bicyclohexyl, 2-(2-methyl propyl)-, trans-2,6-Dodecadien-1-d, 3,7,11-trimethyl-, (E,E)-	0.61
23.	21.720	4,8,12,16-trimethylheptadecan-4-olide 10-methylundecan-5-olide	0.51
24.	23.172	Phthalic acid, di(2-propylpentyl) ester Bis(2-ethylhexyl)phthalate phthalic acid, isoheyl 2-Methylpent-3-yl ester	0.56
25.	27.645	1-propene,3-(2-cyclopentenyl)-2-methyl-1,1-diphenyl-benz(b)-1,4-oxazepine-4(5H)-thione,2,3-dihydro-2,8-dimethyl-1H-Indole,1-methyl-2-phenyl-	4.80
26.	29.635	1-methyl-3-phenylindole Benzo(h)quinolone, 2,4,dimethyl-1,2-Benzisothiazol-3-amine tbdms	2.97

and water-soluble (CAHT) and lipid-soluble antioxidant capacities (CALT) than many plant species (Table 4) [25, 26]. Such data might be useful in the identification of the most beneficial dietary seaweed and in finding new sources of natural antioxidants, such as vitamins E and C and phenolic compounds. In recent years, several algal species have also been reported to prevent oxidative damage by scavenging free radicals and active oxygen, hence possibly preventing cancer cell formation. Therefore, algal species, as alternative sources of natural antioxidative compounds, have attracted much attention from biomedical scientists. There is some evidence that seaweeds contain compounds with a relatively high antioxidant and antigenotoxic activity. Corroborating this, Celikler *et al.* [27] have found lower total phenol and water-soluble antioxidant capacity (CAHT) in *Codium tomentosum* than in *H. musciformis*. On the other hand, *C. tomentosum* has shown higher lipid-soluble antioxidant capacity (CALT) and vitamin E content.

Positive linear correlation between the antioxidant activity and the phenolic contents of the extracts has been reported by Nurul Aili Zakaria *et al.* [28]. Oleic acid

(14.58%) and n-hexadecanoic acid (24.73%) were the major compounds detected, along with the minor compounds of octadecanoic acid, hexadecanoic acid, Z-11-, Cholestane-3,6-dione,(5, 17, 20S)-, 1,2-Benzenedicarboxylic acid, bis(2- methylpropyl) ester, heneicosane, nonacosane and hexacosane. Results of our study matched with a composition study of *Acanthaphora spicifera* from Pakistan in respect to the major compounds of fatty acids detected, octadecadienoic acid (36.05%) and hexadecanoic acid (8.30%) analyzed by Laila, S., [29]. The fatty acids composition of *A. spicifera* from India reported that palmitic acid, arachidonic acid and eicosapentanoic acid as the dominant fatty acids [30].

This difference can be attributed to the climatic and geographical conditions from where the algal was harvested. This is the first phytochemical analysis of volatile components performed by GC-MS reported for *A. spicifera* from Malaysian water, as previous study of this local algal only focusing on the ecological and distribution pattern [31]. The capability of oleic acids and hexadecane that exhibited antimicrobial and antioxidant capabilities have also been reported [32].

In terms of GC identified chemical components, our results varied with the previous studies on *Asparagopsis taxiformis* (Delile) Trevisan by El-Baroty *et al.*, [33]. It could be attributed due to the different geographical region, the developmental stages and seasonal variations.

The activity of marine algae may vary according to the species, seasonality, collection procedures, type of extraction methods and solvents used [34-37].

CONCLUSION

The seaweed *H. musciformis*, a red alga was analyzed for its biochemical and mineral composition. It was found to contain a higher concentration of carbohydrate, protein and mineral and has a moderate concentration of fat and antioxidants. The present study concluded that, marine red seaweed, *H. musciformis* proved to be the most potential seaweed for the development of Pharmaceutical compounds. Our findings have resulted with the following suggestions: *H. musciformis* is red seaweed commonly found during the monsoon and post monsoon season along the Pudumadam coastal area, Gulf of Mannar region in South East Coast of Tamil Nadu, India and can easily be cultivated in coastal areas; the bioactive secondary metabolites can be extracted by cost effective methods.

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