Phytochemical and biochemical composition, in vitro antioxidant potential and GC-MS analysis of red seaweeds *Hypnea valentiae*

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Abstract
The present study focused on pharmaceutical potential of red seaweed Hypnea valentiae. The phytochemical analysis of the methanolic extract of H. valentiae found more phycocompounds which was confirmed by GC-MS analysis. The significant levels of total alkaloid, phenol, tannin and flavonoid were expressed in methanolic extract of H. valentiae. In addition, the potent antioxidant activity of the methanolic extract was efficiently expressed during the total antioxidant activity, free radical scavenging assays of DPPH and hydroxyl denoted as 72.85±0.66 activity at a concentration 50-250µg/mL, 61.44±0.36 activity at a concentration 10-160µg/mL and 83.29±0.38 activity at a concentration 25-125µg/mL respectively.

The results were establishing the potential of the seaweed and antioxidant properties of H. valentiae could be a valuable marine source for developing novel pharmaceutical applications.

Keywords: Secondary metabolites, *Hypnea valentiae*, GC-MS and Antioxidant activity.

Introduction
Seaweeds are marine algae, saltwater-dwelling, simple plants including red, brown and green algae. Most algae have root-like structures called holdfasts that anchor the plant to rocks and other substrates[1] which exhibit a wide spectrum of biological and physiological activities including antimicrobial[39] anticoagulant[37], antiviral[45], antitumor[48], anti-inflammatory[27], antioxidant[34], immune enhancing and hypoglycemic[47]. Seaweed polysaccharide has gained research interest for pharmaceutical, functional food industries and biotechnological applications[33].

In addition, some compounds[31], sulfated[12] and selenylated[50] derivatives of polysaccharides exhibited momentous source of antioxidant activities, higher than the sulfated polysaccharide. Particularly, sulfation is a simple dominant effective to increase biological activity of the polysaccharide molecules[20]. The *H. valentiae* is a sulfated galactone. The polysaccharide isolated from red seaweed mainly consists of sulfated galactose and anhydrogalactose units linked by glycosidic unions[18]. The antioxidant capacity of polysaccharide isolated from various seaweed species has been demonstrated in the literatures[17,24]. It has been reported that polysaccharide naturally exhibits strong secondary antioxidant activity that is comparable to play an important role as oxidative stress often involved as one of the significant sources in the initiation of carcinogenesis.

However, the upcoming cancer therapies have been considered to cause oxidative stress. Antioxidant supplementation is studied as modulator of specific cellular redox signaling mechanisms. Therefore, it is an attractive source of antioxidants and estimates the polysaccharide possible benefits as antioxidant during cancer therapy. In this research, polysaccharide isolated from the *H. valentiae* sample was extracted from methanol for antibacterial activities through the use of five different bacteria for gram positive and gram negative bacteria. Antioxidant activities were also analyzed for sample and standard for their primary and secondary antioxidant activities through the use of different assays and their antioxidant activities were compared against L-ascorbic acid. Lastly, this study attempted to elucidate the basic structure of the purified high-molecular-weight through the use of GC-MS analysis.

Material and Methods
Collection of Sample: The red seaweed *H. valentiae* (Turnur) was collected from Mandapam (Lat. 09o 28’ 177 N, Long. 79o 18’ 536 E), Southeast coastline of Ramanathapuram District, Tamil Nadu, India. The seaweed specimen identification number is 1759. The sample was properly cleaned with sea water to remove all undesired pollutants such as sand particles and epiphytes and then thoroughly cleaned with tap water to discard all salt on the surface. The water was drained away and the seaweed was laid out on blotting paper to absorb any remaining moisture before being shade dried for room temperature in 3 days and crushed into a fine powder.

Extraction of secondary metabolites from *H. valentiae*: A volume of 25g of *H. valentiae* powder was extracted with 250mL of various solvents including benzene, ethyl acetate and methanol for 24 h by using Soxhlet equipment. The extract was collected. The collected supernatant was condensed to dryness under reduced pressure using a rotary evaporator. The partitions was lyophilized and kept in vials at -20ºC.

Evaluation of total alkaloid content: The total alkaloid content of *H. valentiae* methanol extract was determined by

the method of Harborne[14]. 0.5 ml of the sample was taken and kept into a 250ml beaker. 200ml of 10% acetic acid in ethanol was added and allowed to stand for 4hrs. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle for precipitation and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Evaluation of total phenol content: The total phenolic content of *H. valentiae* methanol extract was determined by the method of Siddhuraju et al[38]. To summarize, 200 µL of crude extract (5 mg/mL) was diluted with 3 mL of distilled water and mixed carefully for 3 minutes. In addition, 0.5 mL Folin–Ciocalteu reagent was added with 2 mL of 20% sodium carbonate (w/v). The extract was allowed to stand for 60 minutes in the dark at room condition and absorbance was read at 650 nm (Photocolorimeter-Model-312). The calibration curve represented mg of gallic acid equivalent per dried weight of sample.

Evaluation of total Tannin content: The total tannin content of *H. valentiae* methanol extracts was determined according to the method of Julkunrn-Tiitto[21]. Briefly, 50 µl of extract was mixed with 1.5 ml of 40% vanillin (prepared with methanol) and then 750 µl of HCl was added. Estimation of the tannins was expressed in mg/g dried weight and used as a standard.

Evaluation of total flavonoid content: The total flavonoids content of *H. valentiae* was determined by the method of Chan et al[6]. Each extract was treated with 1 mL of aliquot, 0.1 mL of 1 M potassium acetate and 2.8 mL of methanol. Experimental algal sample was allowed to stand for half an hour at 37°C. The absorbance of combined extract was measured at 415 nm. Estimation of the total flavonoids content was expressed in mg/g and rutin was used as a standard.

Estimation of carbohydrate: The carbohydrate content was estimated by method of Dubois et al[10]. 20 mg of dried seaweeds powder was taken and to this 1 ml of 4% phenol solution and 5 ml of concentrated sulphuric acid were added. After that, they were kept in a dark room for 30 minutes. The color intensity developed was read in a spectrophotometer at 490 nm. Sugar content was calculated by referring to standard D- Glucose and the results have been expressed as mg/g sugar.

Estimation of protein: The carbohydrate content was estimated by Bradford [5].100mg of dry sample is weighed and then grind in mortar and pestle by adding 10ml of distilled water within ice bucket. Filter and centrifuge at 5000 rpm for 5 minutes. Make up the volume of supernatant to 10ml with distilled water. Pipette out 1ml of the extract and add 5ml alkaline mix, allow standing for 10 minutes. Then add 0.5ml of folinciocalteau phenol reagent. Set up the blank and after 30 minutes, the absorbance was read at 500 nm in spectrophotometer.

GC-MS Analysis: The GC-MS analysis of *H. valentiae* methanol extract was performed by 2010 PLUS series Shimadzu using silica column filled with SH-Rxi-%Sil MS (30 m × 0.25 mm ID × 250µm df). The components were separated using helium gas (purity 99.99%) at a 1 mL/min constant flow. The injecting temperature was fixed at 280°C and 1 µL of extract sample was injected into the instrument. The oven temperature was set at 40°C for 2 min, followed by 280°C at the rate of 10°C min^{-1} and held for 3 min at 280°C. The MS detection was completed in 30 min. The spectrums of potential bioactive compounds were identified by comparing them with the 2017 library of the National Institute of Standards and Technology (NIST) [3].

Total antioxidant property: The antioxidant activities of samples were evaluated by the phosphomolybdenum complex formation according to the method of Prieto et al[30]. About 0.5mL sample extracts were added with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were covered with foil and incubated in a water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the reagent blank.

The reported results were mean values expressed as mg AAE /g sample.

Scavenging activity of DPPH: The DPPH-free radical-scavenging activity was assessed with slightly modifications[41]. 3mL of 0.1mM methanolic solution DPPH was prepared and vortexed well. Respective blank sample ascorbic acid was prepared. The discoloration of the sample was measured with a proper blank at 517 nm after incubation for 30 minutes at 30 C in the dark using UV-Vis spectrophotometer (UV-1800, Shimudzu, Torrance, CA). The samples' free radical scavenging activity was calculated as follows:

$$(\%)\ \text{inhibition} = [(A1 - A2)/A1] \times 100$$

where A1 is the absorption of the control and A2 is the absorption of the sample

Scavenging activity of HO: The capacity of the seaweed polysaccharides against the scavenging hydroxyl radical was evaluated by using Fenton's reaction[28]. The solution was incubated at 37° C for 15 min and the presence of yellow color was measured spectrophotometrically at 510 nm against the blank sample. The mixture without the sample was treated as a control. The scavenging activity was calculated by the following equation:

$$(\%)\ \text{inhibition} = \{1 - [(A1 - A2)/A0]\} \times 100$$

where A0 control, A1 is the absorption of the sample and A2 is absorption without sodium salicylate

Results and Discussion

Qualitative phytochemical analysis: Preliminary phytochemical constituents of eight different chemical compounds (alkaloids, flavonoids, phenols, saponins, tannins, steroids, glycosides, terpenoids and anthraquinone) were tested in three different extracts of benzene, ethyl-acetate and methanol fraction as shown in table 1. The phytochemical constituents of crude methanol extract of *H. valentiae* were present in the extract. Glycosides, terpenoids and anthraquinone were not present in the extracts and fractions. Among the three extract tested, benzene extract showed the presence of alkaloids, flavonoids, phenols, saponins and tannins of *H. valentiae*. Ethyl acetate extract showed the presence of alkaloids, flavonoids, phenols, saponins, tannins and terpenoids. The presence of the phytochemicals depends upon the solvent medium used for extractions. In general, seaweed extracts especially phenolic compounds have unique physiological and biological activities including antioxidant properties that make them valuable therapeutic applicants.

Phenolic were also responsible for the antimicrobial, anti-inflammatory, antiapoptosis, anti-viral, anticancer as well as for inhibition of angiogenesis and cell proliferation activities[7]. Alkaloids have indicated the biological activity against the antimicrobial properties and prevent the cell division[44]. Flavonoids are reported to show the activity against the antimicrobial, antiviral, antioxidant and anti-inflammatory activities[2]. Phenols possess numerous biological properties which include anti-inflammatory and anti-feedent[7]. Saponins are found in the insecticidal, antimicrobial and antiparasitic of *sargassum wightii*[43]. Tannins were found in antiviral, antibacterial inhibition[49]. Steroids have been reported in the *Gracilaria debilis* for antimicrobial and antiparasitic properties[2]. The presence of methanol extract of *H. valentiae* can be used in medicinal properties.

Quantitative phytochemical Analysis: Seaweeds found to be rich in medicinal value in secondary metabolites such as alkaloids, phenols, flavonoids and tannin having potential medicinal properties. The highest total alkaloids content was 6.89±0.40 Mg/10g, tannins content was 3.44±0.48 Mg/10g, flavonoids was 2.04±0.05 Mg/10g content and phenols content being 0.71±0.06 Mg/10g. The total phenol content enhances the phytochemical constituent in algae such as *Laminaria, Undaria, Scytosiphon and Tunbinaria*[13]. Alkaloids, flavonoids, phenols and tannins have stimulated the antimicrobial[39], antiviral[22], antioxidant[19] and anti-inflammatory activities[27].

Biochemical analysis: The biochemical analysis of carbohydrate and protein content of *H. valentiae* seaweed is presented in table 2. The carbohydrate content was 59.42±0.07 Mg/10g and protein content was 29.60±0.08 Mg/10g.

Table 1
Qualitative phytochemical analysis of *H. valentiae*

Name of the Compounds	Benzene	Ethyl- acetate	Methanol
Alkaloids	+	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Saponins	+	+	+
Tannins	+	+	+
Steroids	-	-	+
Glycosides	-	-	-
Terpenoids	-	+	-
Anthraquinone	-	-	-

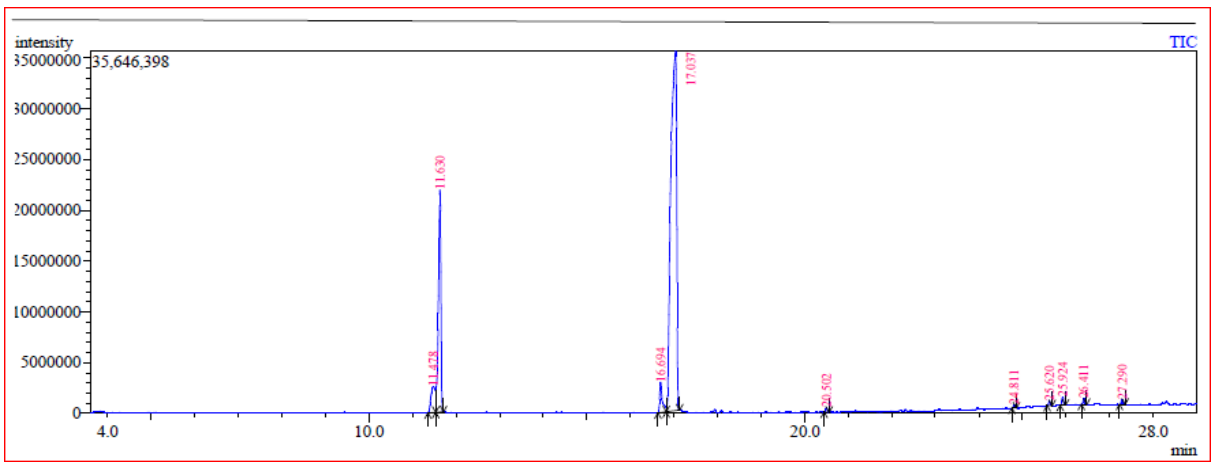


Figure 1: GC-MS analysis in Methanol extract of *H. valentiae*

Table 2
Quantitative and Biochemical analysis of methanol extract *H. valentiae*
Alkaloid, Phenol, Tannin, Flavonoid, Carbohydrate and Protein

Quantitative analysis	Content Mg/10g
Alkaloids	6.89±0.40
Flavonoids	2.04±0.05
Phenol	0.71±0.06
Tannin	3.44±0.48
Biochemical Analysis	
Carbohydrates	59.42±0.07
Protein	29.60±0.08

Table 3
GC-MS analysis of methanol extract of *H. valentiae*

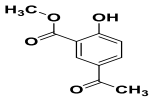
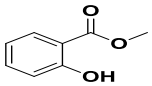
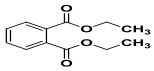
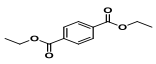
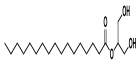
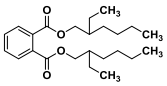
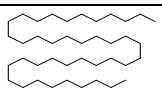
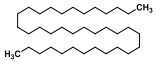
S.N.	Compound	RT	Molecular weight and Formula	Molecular stucture	Area %	Bioactivity
1	Benzoic acid, 2-hydroxy-, methyl ester	11.478	$C_8H_8O_3$ 152		3.83%	Antimicrobial
2	Methyl salicylate	11.630	$C_8H_8O_3$ 152		19.27%	Analgesic
3	Diethyl Phthalate	16.694			2.78%	Anti-microbial activity
4	1,2-Benzenedicarboxylic acid, diethyl ester	17.037	$C_{12}H_{14}O_4$ 222		72.38%	Neurodegenerative disorders, anti-cancer activity.
5	Hexadecanoic acid, methyl ester	20.502	$C_{19}H_{38}O_4$ 330		0.23%	Antioxidant activity Hemolytic, pesticide, flavour, hypocholesterolemic , Antiandrogenic Alpha reductase inhibitor.
6	bis(2-ethylhexyl) phthalate	25.924			0.18%	Antioxidant activity, Antibacterial activity
7	Tetracontane	26.411	$C_{40}H_{82}$		0.26%	anti-inflammation and analgesic activities
8	Hexatriacontane	26.419	$C_{36}H_{74}$ 506		0.47%	Antiinflammatory, analgesic activity

Table 4
Antioxidant activity of *Hypnea valentiae* methanol extract

Extract/ Positive control	Total	DPPH	HO
	(50-250µg/mL)	(10-160µg/mL)	(25-125µg/mL)
Methanol *Hypnea valentiae*	72.85±0.66	75.08±0.67	72.85±0.66
L-Ascorbic acid	30.07±0.73	84.06±0.77	84.95±0.77

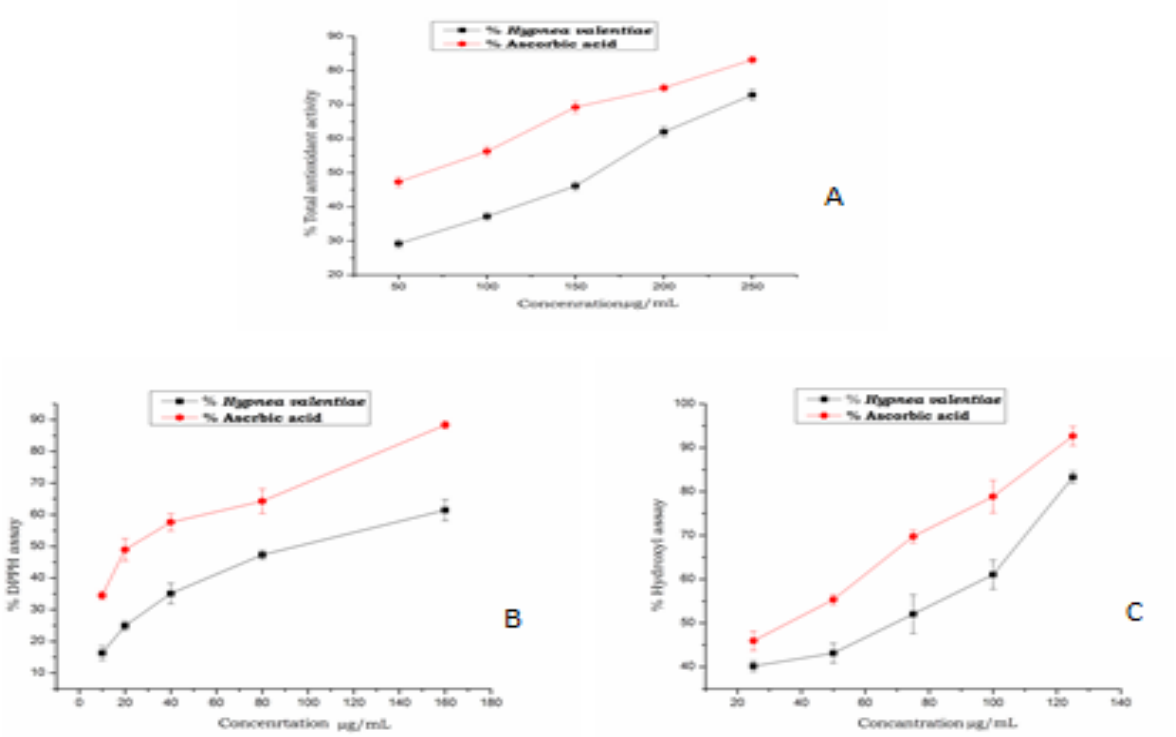


Figure 2: Antioxidant assay (A) Total antioxidant activity (B) DPPH assay and C. Hydroxyl assay

GC-MS Analysis: The GC-MS analysis was carried out in the methanol extract of *H. valentiae* in different biological compounds shown in fig. 1. The mass spectrum of retention time of all the compounds was identified in *H. valentiae*. The results showed that the seaweed extracts contained 10 bioactive compounds such as benzoic acid, 2-hydroxy-, methyl ester, methyl salicylate, diethyl phthalate, 1,2-benzenedicarboxylic acid, diethyl ester, hexadecanoic acid, methyl ester, bis(2-ethylhexyl) phthalate, tetracontane and hexatriacontane.

Antioxidant activity

Total antioxidant property: The total antioxidant activity was determined to evaluate antioxidant capacity of seaweed methanol extracts from sulfated polysaccharide *H. valentiae*. The extract demonstrated the inhibition of total antioxidant activities (50 -250 µg/mL) of the exhibited activities in 72.85±0.66. In the present study, ABTs assay was consistently higher in inhibition of methanol extracts of *H. valentiae.* The results were also comparable to similar research work reported in *Turbinaria ornata*, showing that the higher total antioxidant activity is a significant indicator of its potential antioxidant activity[32]. The total antioxidant activity of seaweed is functional of sulphate groups, galactons and carboxylic acid attributed due to the antioxidant activity from *G. corticata* and *G. edulis* [4].

Scavenging activity of DPPH: The DPPH radical scavenging assay to determine the inhibition demonstrated activity of polysaccharide to increase significantly with the concentrations of *H. valentiae*. Carbohydrate extracts also exhibited DPPH radical scavenging activity which is very harmful to cellular components as a precursor of more reactive species such as single oxygen and hydroxyl radicals *Undaria pinnatifida*[25]. DPPH is a reduced form of molecular oxygen created by receiving one electron, which is less active and has less harmful effect to the organism. The extract had very stronger scavenging ability on DPPH radicals such as ABTs and nitric oxide[9]. The *Sargassum thunbergii* is more active in inhibiting radical scavenging activity of polysaccharide compounds and that the sulfate content affects their antioxidant activity[23]. The radical ability of the polysaccharide demonstrated that the sulfate content affected their antioxidant properties. Radical scavenging assay was a weak oxidant in most organisms inducing pathological disease *Laminaria japonica*[46].

Scavenging activity of HO: The hydroxyl radical scavenging capability of *H. valentiae* methanol extracts solution on hydroxyl radical was determined as described previously by Leong et al[28]. This assay was used to test the ability of the antioxidative compounds (10 -100 µg/mL) of the exhibited activities in 83.29±0.38 as in table 2. The polyphenolic chemicals from seaweeds have demonstrated to be highly effective in antioxidant properties against free radicals. Polyphenols with their antioxidant activity were higher in the sample.

The hydroxyl is a reactive free radical made by phagocytes and endothelial cells to produce more reactive species like peroxy-nitrite which can decompose to form OH radical. However, the level of hydroxyl was significantly reduced by the *G. corticata* and *G. edulis* extracts in the study[4].

Conclusion

The marine macro algae contain large amounts of physiologically and biologically activities. The methanolic extracts demonstrated that the red seaweeds *H. valentiae* is valuable source of secondary metabolite and therefore has high potential to treat a wide range of many diseases. *H. valentiae* has different levels of antioxidant properties. GC-MS analysis revealed the presence of numerous bioactive metabolites such as benzoic acid, 2-hydroxy-, methyl ester, methyl salicylate, diethyl phthalate in both the red seaweeds.

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