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Gas Chromatography and Mass Spectrum Analysis and *In Vitro* Antibacterial Activity of Macro Alga *Hypnea Valentiae*

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Abstract: ***In the present study Hypnea valentiae was collected along the shore of Mandapam and was identified and authenticated. To analyse the methanol extract of marine red macro alga species H. valentiae using Gas chromatography-Mass spectrometry (GC-MS) and in vitro antibacterial activity. Gas chromatography and mass spectrometry analysis of seaweed extract injected with instrument GC-MS-QP 2010 [SHIMADZU] and antibacterial activity of human pathogenic bacteria. The methanol extracts of Hypnea valentiae was identified 27 bioactive compounds which were major compound such Hexadecanoic acid-methylester, 13-Docosenamide, (Z) and Eicosane (RT, 21.07, 34.46, 16.51; 29.10%, 10.27%, 10.24%) Cholest-5-en-3-ol, 24-propylidene-, (3á)-, 9-Octadecenoic acid (Z)-, methyl ester (CAS) (RT39.09, 24.38; 9.00%, 8.07%) Hexadecanoicacid (CAS) - Methyl tetradecanoate (RT 21.89, 17.04; 4.74%, 3.30%). In India, seaweeds have been utilized since the ancient times as source of human food, animal feed, fertilizer, as well as herbicides. In conclusion of the appreciable antimicrobial activity and thus have great potential solvent to extract bioactive compounds from the natural sources for current clinical and pharmaceutical importance.***

Keywords: Macro algae, Hypnea valentiae, GC-MS analysis, Antibacterial activity.

I. INTRODUCTION

Marine macroalgae (seaweeds) are renewable and promising resources of numerous biologically active compounds. These chemical compounds are potential sources of commercial as well as therapeutic impact of seaweeds are still unaware. A total of ten thousand five hundred seaweed species are reported worldwide and 865 species of the seaweeds are found in India. Seaweeds are important for the coastal ecosystems as an ocean producer. The seaweed cultivation occupies the third-largest aquaculture production worldwide. It has a portion of human foods exceeding a hundred years (Bjerregaard *et al*., 2016). The worldwide seaweed production contributes approximately 20% of total global marine aquaculture production (Divyagnaneswari *et al*., 2007). Marine algae are rich sources of elements activities, such as antibiotic, antiviral, antioxidant, vary from species to species. For example, large quantities antifouling, anti-inflammatory, cytotoxic and antimitotic activities (Salvador *et al*., 2007).

H. valentiae belongs to the family Hypneace whose plants are bushy, cylindrical, 10-30cm high, purplish green in color, cartilaginous, much branched, branches irregular, giving a bushy look (Hayee-Memon and Shameel, 1996). It has been reported to possess k-carrageenan (Andrade *et al*., 2000). Carrageenan is extensively used as a food additive in a wide range of food products. Marine resources are an unmatched reservoir of biologically active natural products, many of which exhibit structural features that have not been found in terrestrial organisms (Saritha *et al*., 2013). There are numerous reports on compounds derived from macro algae with broad ranges of biological activities, such as the antimicrobial, antiviral, anti-tumour, anti-inflammatory, and neurotoxic (El-Din and El-Ahwany, 2015). Therefore, in the present study was subjected to assess the Gas chromatography and mass spectrum analysis and in vitro antibacterial, activity of *Hypnea valentiae*.

II. MATERIALS AND METHODS

A. Sample Collection

The seaweed of red macro algae *Hypnea valentiae* (Figure 1) was collected from the intertidal shallow zone at depth of 0-1 m at Mandapam, Ramanathapuram, Tamil Nadu. The alga was obtained from the Mandapam coast, Gulf of Mannar region, Rameswaram (Latitude: 9°16’32.56” N and Longitude: 79°7’25.03” E) along the southern regions of Tamil Nadu. The sample of seaweed was identified self and binomially by Botanical Survey of India (Southern part Coimbatore, Tamilnadu, India) and voucher specimen (BSI/SRC/5/23/2018/Tech.1383) was deposited at the Herbarium Department of zoology, Kongunadu Arts and Science College (Autonomous), Coimbatore, Tamilnadu, India.

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Figure 1. Seaweed (Red Algae)

B. Extraction of the Material

50g of *Hypnea valentiae fine* powder was packed with Whatman No.1 filter paper and placed in Soxhlet apparatus along with solvent methanol. The residues were collected and dried at room temperature, 30˚C after which yield was weighed and then performed to activity.

C. Gas Chromatography and Mass Spectrum Analysis

5 ml of methanol extract was evaporated to dryness and reconstituted into 2 ml methanol. The extract was then subjected to GC-MS analysis. Chromatographic separation was carried out with instrument GC-MS-QP 2010 [SHIMADZU] instrument with Db 30.0 column (0.25µm diameter × 0.25um thickness). The oven temperature was programmed from 70 °C (isothermal for 5 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 35 min isothermal at 280°C. Mass spectra was taken at 70 eV; a scan interval of 0.5 s and Scan range from 40–1000 m/z. Helium was used as carrier gas at 99.999% pressure with flow 1.0 ml/min and electronic pressure control on. Sample was dissolved in methanol and injected automatically.

D. Analytical Condition

Injection temperature at 240^0C, interface temperature at 240^0C and ion source temperature at 70^0C were determined. Injection was performed in split less mode.

E. Identification of Compounds (Data Analysis)

The mass spectra of compounds in samples were obtained by electron ionization (EI) at 70 eV and the detector operator in scan mode from 40 to 1000 m/z atomic mass units. Identification based on the Molecular weight, Molecular formula, Retention time and peak area %.

F. Identification of Compounds

Identification was based on the active principles with their Retention time (RT), Molecular formula (MF), Molecular weight (MW) and concentration (peak area %). It is done in order to determine whether this plant species contains any individual compound or group of compounds which may substantiate its current commercial and traditional use as herbal medicine, in addition to determine the most appropriate methods of extracting these compounds. These results will consequently be discussed in the light of their putative biological and therapeutic relevance.

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G. In vitro Antibacterial Activity

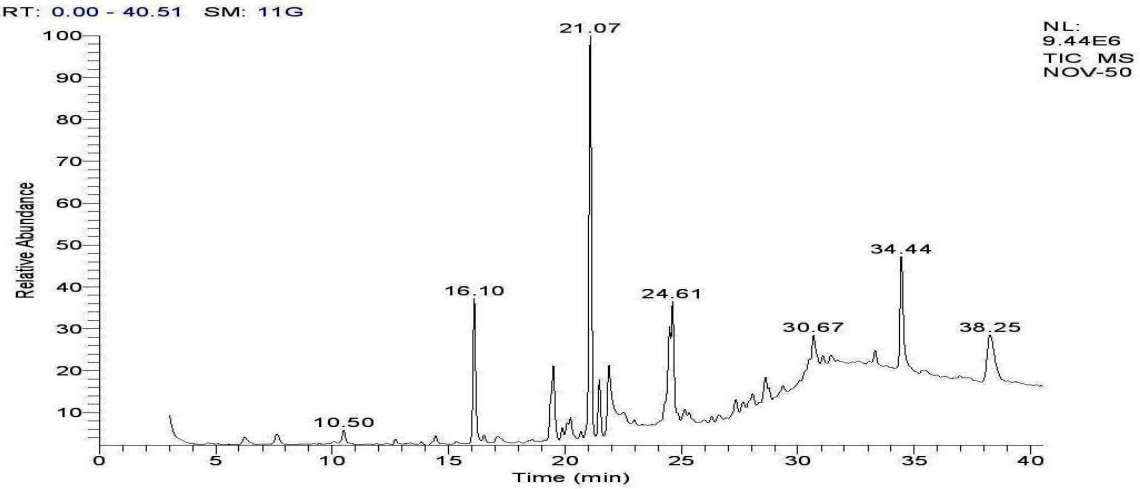
In vitro antibacterial activity of *Hypnea valentiae* extracts were determined using disc diffusion method. Two-gram positive bacteria *Streptococcus faecalis, Bacillus subtilis* and four-gram negative bacteria; *Staphylococcus aureus, Klebsiella pneumonia, Salmonella paratyphi, Pseudomonas aeruginosa*, were used for this study. The bacteria were sub cultured on nutrient broth, incubated at 37°C for 24 h and stored at 4°C in the refrigerator to maintain stock culture. Culture plates were prepared with 20 ml of sterile nutrient agar medium. The test cultures were swabbed on the top of the solidified agar media and allowed to dry for 10 minutes. The discs of 6 mm diameter were prepared from Whatman filter paper No. 1 and sterilized. The sterilized discs were then impregnated with respective (petroleum ether, chloroform, methanol and aqueous) extracts and placed on the surface of the medium and left for 30 minutes at room temperature for compound diffusion (Pandithurai *et al.*, 2015). Discs impregnated with tetracycline (1 mg/ml) were used as positive control. The plates were incubated for 24 h at 37°C. The diameter of the zone of inhibitions was measured by measuring scale in millimetre (Thanigaivel *et al*., 2012). The sensitivity of the microorganisms to plant extract was determined by measuring the size of inhibitory zones on the agar surface around the discs (Vairappan *et al*., 2008).

H. Statistical Analysis

All analyses were carried out in triplicate and the data were reported as mean where there was significance of the difference between means was determined by Duncan's multiple range test ($p<0.05$) using statistical.

III. RESULTS AND DISCUSSION

The GC-MS analysis of the crude extract revealed that the main compound were Hexadecanoicacid-methylester,13-Docosenamide, (Z) and Eicosane (RT, 21.07, 34.46, 16.51; 29.10%, 10.27%, 10.24%) Cholest-5-en-3-ol, 24-propylidene-, (3á)-, 9-Octadecenoic acid (Z)-, methyl ester (CAS) (RT39.09, 24.38; 9.00%, 8.07%) Hexadecanoicacid (CAS) - Methyl tetradecanoate (RT 21.89, 17.04; 4.74%, 3.30%) involved in the biological activity. The *H. valentiae* based on spectral data by GC-MS analysis was found to be a mixture of volatile compounds. 30 peaks were observed with retention times as presented in **Table 1, 2 and Graph. 2**. The GC-MS analysis of the crude extract revealed that the main constituent were Hexadecanoic acid-methyl ester, Hexadecen-1-ol-tetramethyl (RT21.07, 24.61; 26.66%, 12.17%) 3-Heptadecene,(Z)-,13-Docosenamide,(Z)- (RT 16.10, 34.44; 9.60%, 7.88%) Hexadecanoicacid-methyl ester-Ethyl tridecanoate biosynthesis occurs only in plants, it would be useful to and 2-Pentadecanone, 6, 10, 14-trimethyl- Retinoic acid, methyl ester (CAS) (RT 19.50,38.25; 6.18%, 5.10%) may be elaborate in biological activity (Bhaskar *et al*., 2004). Many species of macro algae possessed main constituents such as tetradecnoic acid, hexadecanoic acid, octadecanoicacid methyl ester, which may reveal antagonism against bacteria. The capability of palmitic acid, oleic acids and hexadecane that exhibited antioxidant and antimicrobial abilities have also been reported by Plaza *et al*., (2010). Most of the identified components have been reported to possess antimicrobial activity that could be responsible for the antifungal potential reported in the present study (Shobier *et al*., 2016). The prevailing compounds are of this (alcoholic compound, aromatic ether compound, flavonoid, amino compound, steroid, phenolic halogen, alkaloid, fluro-benzoic acid ester, diterpenoids and Ketimines). The identified compounds have the property of antifungal, antioxidant and antimicrobial activity. Antimicrobial activity depends on both algal species and the solvents used for their extraction (Radhika *et al*., 2012).



Graph: 1. GC-MS Chromatogram of the Red alga *Hypnea valentiae*

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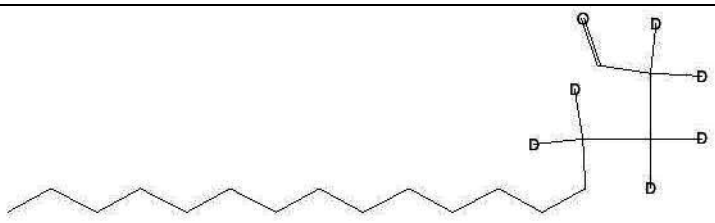
Table: 1. Identification of chemical compounds by using Gas Chromatography and Mass Spectroscopy analysis of methanolic extracts of *H. valentiae.*

S. No	Compound name	Retention Time (RT)	Peak Area (%)	Molecular formula (MF)	Molecular weight (MW)
1	N-BZ-2 amino cinnamate	6.24	0.80	$C_{17}H_{17}NO_2$	267
2	Dotriacontane (CAS)	7.63	0.84	$C_{32}H_{66}$	450
3	Tetradecane (CAS)	10.50	1.00	$C_{14}H_{30}$	198
4	Docosane (CAS)	14.43	0.74	$C_{22}H_{46}$	310
5	3-Heptadecene, (Z)-	16.10	9.60	$C_{17}H_{34}$	238
6	Methyl 16-hydroxy hexadecanoate	17.08	0.72	$C_{17}H_{34}O_3$	286
7	2,2,3,3,4,4 hexadeuterooctadecanal	18.59	0.46	$C_{18}H_{30}D_6O$	268
8	2-Pentadecanone, 6,10,14-trimethyl-	19.50	6.18	$C_{18}H_{36}O$	268
9	Phytol, acetate	19.87	0.54	$C_{22}H_{42}O_2$	338
10	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	20.24	1.93	$C_{20}H_{40}O$	296
11	9-Hexadecenoic acid, methyl ester, (Z)-(CAS)	20.69	0.46	$C_{17}H_{32}O_2$	268
12	Hexadecanoic acid, methyl ester	21.07	26.66	$C_{17}H_{34}O_2$	270
13	5,6,11,12-Tetrahydro-6-methyl-5,11,12-trioxopyrido[2,3-b] acridine	21.48	3.99	$C_{17}H_{10}N_2O_3$	290
14	Hexadecanoic acid (CAS)	21.89	4.93	$C_{16}H_{32}O_2$	256
15	Hexadecanoic acid, 2,3-dihydroxypropyl ester (CAS)	22.53	0.67	$C_{19}H_{38}O_4$	330
16	2-Hexadecen-1-ol,3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS)	24.61	12.17	$C_{20}H_{40}O$	296
17	Tetraneurin - A – diol	25.14	1.90	$C_{15}H_{20}O_5$	280
18	à-D-Glucofuranose, 6-O-(trimethylsilyl)-, cyclic1,2:3,5-bis (butyl boronate) (CAS)	26.59	0.61	$C_{17}H_{34}B_2O_6Si$	384
19	Docosanoicacid,8,9,13-trihydroxy-, methyl ester (CAS)	27.33	1.31	$C_{23}H_{46}O_5$	402
20	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	27.64	0.62	$C_{57}H_{104}O_6$	884
21	L-Tryptophanamide,1-methyl- 5-oxo-L-prolyl-N,1-dimethyl-L-histidyl-N,N,Nà,1-tetramethyl-	28.06	1.41	$C_{29}H_{39}N_7O_4$	549
22	7-Methyl-Z-tetradecen-1-ol acetate	28.61	2.78	$C_{17}H_{32}O_2$	268

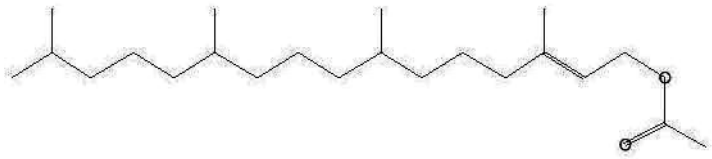
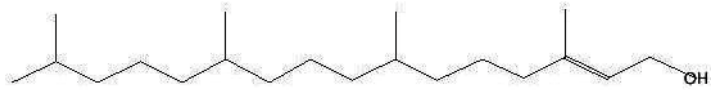
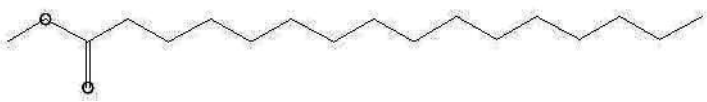
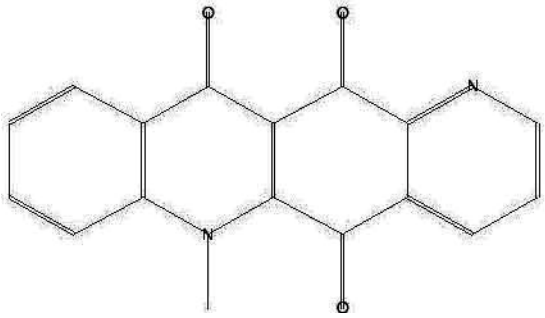
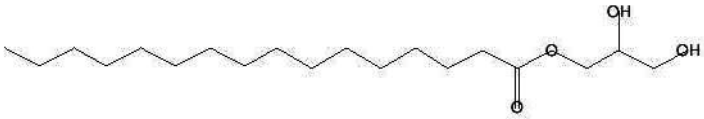
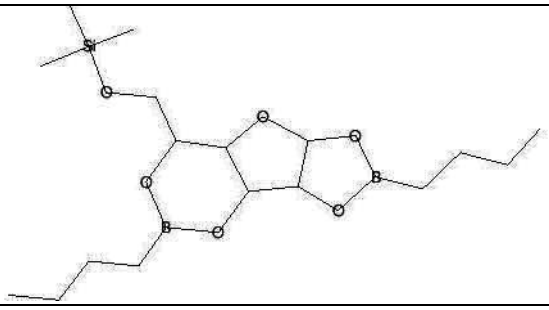
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23	Lucenin 2	29.36	0.71	$C_{27}H_{30}O_{16}$	610
24	Hexadecanoic acid, 1(hydroxymethyl)-1,2-ethanediyl ester (CAS)	30.67	3.22	$C_{35}H_{68}O_5$	568
25	Lucenin 2	31.08	0.54	$C_{27}H_{30}O_{16}$	610
26	Lucenin 2	31.40	0.72	$C_{18}H_{32}O_4$	312
27	Methyl7-ethyl-10-hydroxy-11-droxy(18o)-3,11-dimethyl-2,6-tridecadienoate	33.32	0.93	$C_{22}H_{43}NO$	337
28	13-Docosenamide, (Z)-	34.44	7.88	$C_{27}H_{30}O_{16}$	610
29	Lucenin 2	35.48	0.58	$C_{21}H_{30}O_2$	314
30	Retinoic acid, methyl ester (CAS)	38.25	5.10	$C_{18}H_{32}O_4$	312

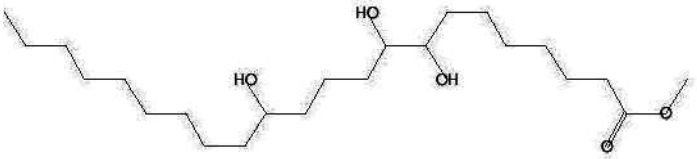
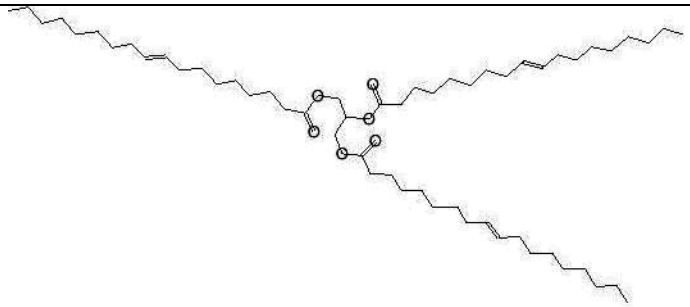
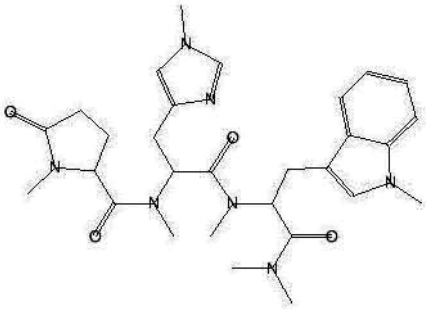
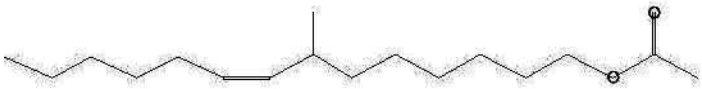
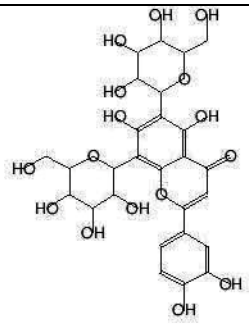
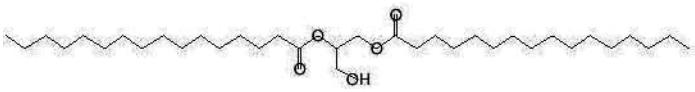
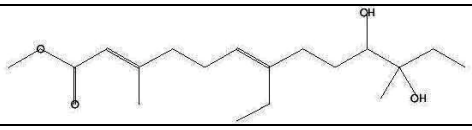
Table: 2. Structure and Nature of the compound Red alga *H. valentiae*.

S. No	Compound	Structure	Nature of the compound
1	N-BZ-2 amino cinnamate	----------	Methyl ester of cinnamic acid
2	Dotriacontane (CAS)		Alkanes. hydrocarbon lipid molecule
3	Tetradecane (CAS)		Alkanes. hydrocarbon lipid molecule
4	Docosane (CAS)		Aliphatic acyclic compounds, alkanes.
5	3-Heptadecene, (Z)-		Alkane.
6	Methyl 16-hydroxy hexadecanoate		Carboxylic ester derivatives of a fatty acid.
7	2,2,3,3,4,4 hexadeuterooctadecanal		Oxosteroid
8	2-Pentadecanone, 6,10,14-trimethyl-		Sesquiterpenoid

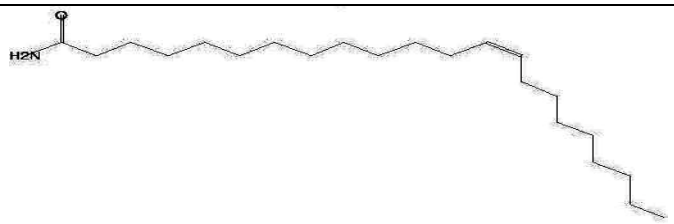
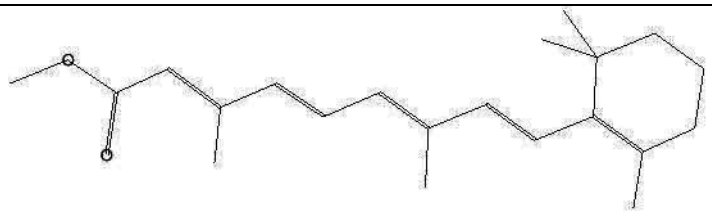
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9	Phytol, acetate		Acyclic diterpenoids
10	3,7,11,15-Tetramethyl-2-hexadecen-1-ol		Acyclic diterpenoids
11	9-Hexadecenoic acid, methyl ester, (Z)- (CAS)		Unsaturated fatty acid
12	Hexadecanoic acid, methyl ester		Palmitic acid methyl ester
13	5,6,11,12-Tetrahydro-6-methyl-5,11,12-trioxopyrido[2,3-b] acridine		Pyridoacridine
14	Hexadecanoic acid (CAS)		Palmitic acid
15	Hexadecanoic acid, 2,3-dihydroxypropyl ester (CAS)		Monopalmitin
16	2-Hexadecen-1-ol,3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS)		Acyclic diterpenoids
17	Tetraneurin - A – diol	----------	Aliphatic heteromonocyclic compounds
18	à-D-Glucofuranose, 6-O-(trimethylsilyl)-, cyclic1,2:3,5-bis (butyl boronate) (CAS)		Phenoxy compound

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19	Docosanoicacid,8,9,13-trihydroxy-, methyl ester (CAS)		O-glycosyl compound, Disaccharide
20	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-		Unsaturated fatty acid
21	L-Tryptophanamide,1-methyl- 5-oxo-L-prolyl-N,1-dimethyl-L-histidyl-N,N,Nà,1-tetramethyl-		Amino acid
22	7-Methyl-Z-tetradecen-1-ol acetate		Terpenoid; Ester
23	Lucenin 2		Flavonoid
24	Hexadecanoic acid, 1(hydroxymethyl)- 1,2-ethanediyl ester (CAS)		Diacylglycerol-3-phosphate, Fatty acid ester
25	Methyl7-ethyl-10-hydroxy-11-droxy(18o)-3,11-dimethyl-2,6-tridecadienoate		Ketimines

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26	13-Docosenamide, (Z)-		Fatty amides
27	Retinoic acid, methyl ester (CAS)		Retinoids, steroid

A. In vitro Anti Bacterial Activity

The in vitro antibacterial activity of petroleum ether, chloroform, methanol and aqueous extracts of *H. valentiae* showed varied activity against different pathogens treated **(Table 3).** It was found that the zones of inhibition were ranged between 6.7 to 33.6 mm. Gram positive bacterial strain, *Streptococcus faecalis* showed highest inhibition zone (29.3±1.2) in methanol extract followed by aqueous extracts (11.0±1.2) and chloroform (9.3±0.5). The gram-negative bacteria, *Staphylococcus aureus* was maximum inhibited (32.0±1.6) by the methanol extract compared to all other extracts and control. However, chloroform extract showed next higher inhibition zone (15.6±0.2). Though the methanol extract exhibited maximum inhibited zone in all the bacteria, *Salmonella paratyphi* and *Pseudomonas aeruginosa* were inhibited maximum by the standard, tetracycline. Whereas, aqueous extract reacted more on *Salmonella paratyphi* and *Pseudomonas aeruginosa* than the methanol extract and showed similar inhibition zone (18.3±1.2). Lowest inhibition zones were observed in all the bacterial strains when petroleum ether extract was used. The acetone extract showed greater activity against gram-positive organisms than gram-negative organisms. Higher antibacterial activity of the extract may be owing to the greater solubility of the metabolites of the plants in this organic solvent (Vinoth *et al.*, 2015; Pandithurai *et al.*, 2015).

Table. 3: Evaluation of *In-vitro* antibacterial activity of various solvent extracts of Red algae *Hypnea valentiae*.

Bacterial Strains	ZONE OF INHIBITION (mm)				
	Standard (Tetracycl	Petroleum ether	Chloroform Extract	Methanol Extract	Aqueous Extract
Gram Positive					
Bacillus subtilis	12 ± 0.8^{e}	8.7 ± 1.2^{b}	9.0 ± 1.6^{d}	13.3 ± 1.2^{c}	10.3 ± 1.2^{d}
Streptococcus faecalis	24.7 ± 1.7^{b}	7 ± 0.8^{de}	9.3 ± 0.5^{cd}	29.3 ± 1.2^{b}	11.0 ± 1.2^{c}
Gram Negative					
Klebsiella pneumoniae	23.3 ± 1.2^{bc}	7.3 ± 1.2^{d}	8 ± 0.8^{e}	28.7 ± 1.2^{bc}	13.6 ± 1.2^{b}
Pseudomonas aeruginosa	21 ± 1.6^{c}	10.3 ± 0.9^{a}	9.7 ± 1.7^{b}	12.3 ± 1.7^{cd}	18.3 ± 1.2^{a}
Salmonella paratyphi	15.6 ± 0.9^{d}	8 ± 0.8^{cd}	9.6 ± 1.2^{bc}	13.3 ± 1.2^{c}	18.3 ± 1.2^{a}
Staphylococcus aureus	26.6 ± 1.2^{a}	6.6 ± 0.5^{e}	15.6 ± 0.2^{a}	32.0 ± 1.6^{a}	7.3 ± 1.2^{e}

Values are mean±SD (n=3); Mean values followed by different superscripts in a column are significantly different ($P<0.05$) according to Duncan's multiple range tests (DMRT).

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IV. CONCLUSION

It can be concluded that marine macro algae are a rich source of structurally novel and biologically active metabolites. Secondary or primary metabolites produced by these macro algae may be potential bioactive compounds of interest in the pharmaceutical industry and medicinal compounds. Bioactive compounds found in seaweeds await a major breakthrough for their potential application as natural antioxidants in different food and pharmaceutical products.

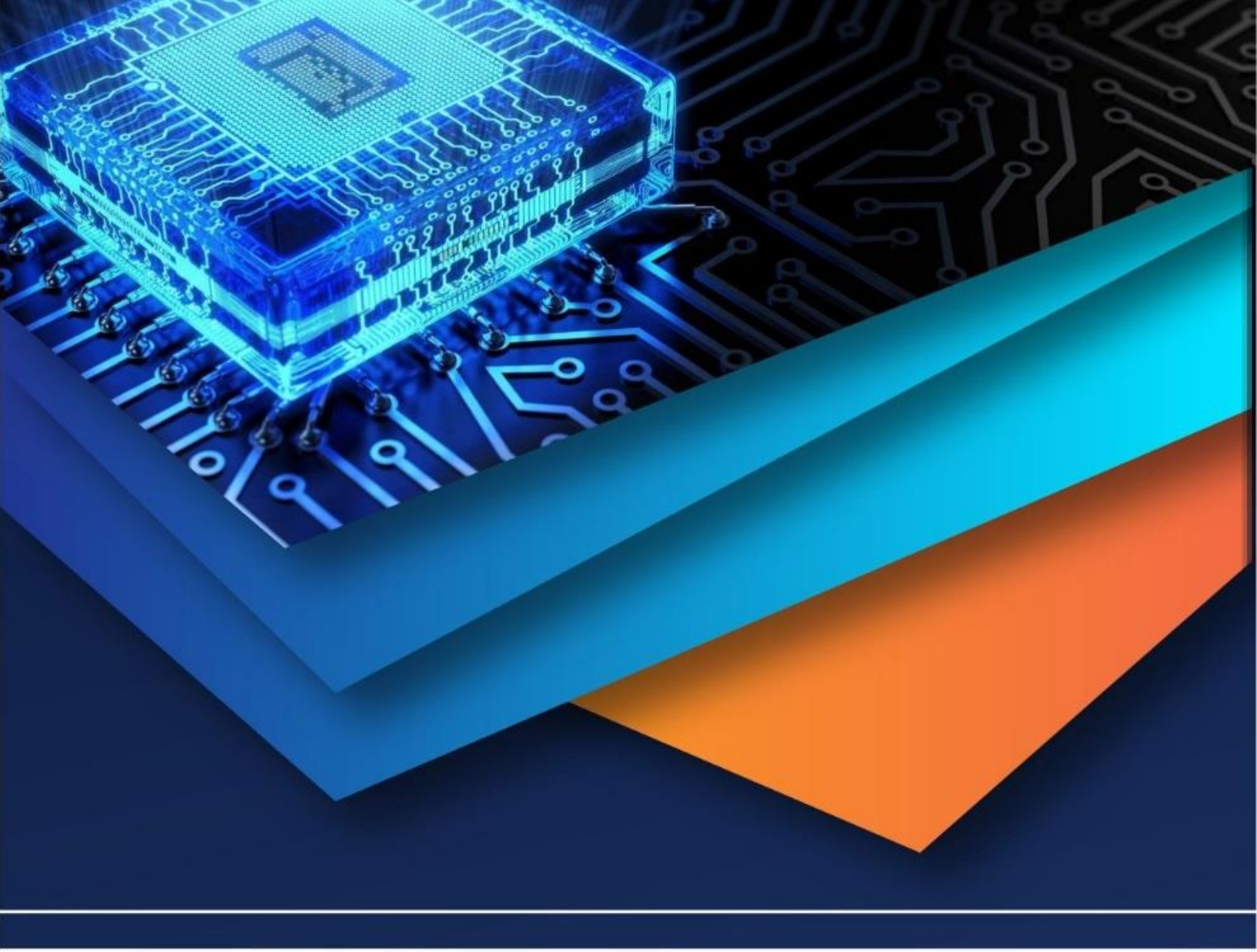
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