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IDENTIFICATION OF ANTIOXIDANT COMPOUND CHOLEST-5-EN-3-OL FROM CHLOROFORM EXTRACT OF *GRACILARIA FOLIIFERA* USING GC-MS ANALYSIS

Jenifer P.[1], C. P. Balakrishnan[1]* and S. Chidambaram Pillai[2]

[1]Department of Botany, Aditanar College of Arts and Science, Virapandianpatnam, Tiruchendur - 628 216, Tamil Nadu, India.

[2]PG and Research Department of Botany, V.O. Chidambaram College, Tuticorin- 628 008, Tamil Nadu, India.

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***Corresponding Author**
C. P. Balakrishnan
Department of Botany, Aditanar College of Arts and Science, Virapandianpatnam, Tiruchendur - 628 216, Tamil Nadu, India.

ABSTRACT

The present study deals with identification and evaluation of antioxidant compound Cholest-5-en-3-ol by using GC-MS analysis from chloroform extract of marine red algae *Gracilaria foliifera* J. Agardh of Manapad coast, Tamil Nadu. *In-vitro* antioxidant analysis like DPPH, H_2O_2, OH, NO radical and total antioxidant activity also carried out by using standard methods. The GC-MS analysis has shown the presence of different phytochemical compounds in the chloroform extract of *G. foliifera*. Over 30 phytochemical compounds have been identified, among these, the phytochemical compound Cholest-5-en-3-ol with retention time (RT) 31.79 min and peak area 5.08% (Molecular formula $C_{27}H_{46}O$ and Molecular weight 386). The similar studies from plants were agreed that compound Cholest-5-en-3-ol is activity of antioxidant. *In-vitro* antioxidant result indicated that chloroform extract of *G. foliifera* exhibited significant value were compared with standard.

KEYWORDS: Seaweed, *G. foliifera*, phytochemical screening, GC-MS, *in-vitro* Antioxidant.

INTRODUCTION

Primitive plants especially thallophytes are the simplest form and most of them are aquatic habitat. Among these marine macro algae are the dominant once to majorly occupy the seashore by free floating or attached on the rocky substratum. Now a day the aware of natural

products from these plants is plenty meantime that natural constituents active against various ailments. Marine macroalgae are also rich in bioactive compounds with anti-inflammatory, antimicrobial, antitumoral, antiviral and antioxidant activities.[1] The secondary metabolites such as carotenoids, tocopherols, terpenes and phenolic compounds can be considered natural antioxidants and with several potential applications.[2-4] An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is an essential biological process of energy production in many living organisms. The antioxidants have main roles in scavenging the free radicals, maintaining the cell integrity, slow down ageing and prevent the development of complications associated with oxidative stress- related diseases and cancer.[5] The purpose of the present study was to identification and evaluates the antioxidant compound of chloroform extract of *G. foliifera* by using GC-MS analysis and various methods of *in-vitro* antioxidant activity.

MATERIALS AND METHODS

Collection of seaweed

Marine red algae *Gracilaria foliifera* J. Agardh was collected from Manapad coast of Tamil Nadu, India (8.3775°N; 78.0522°E) at low tide. Specimen was washed thoroughly in seawater to remove extraneous matter such as epiphytes and sand. After collection, fresh samples were taken into plastic jar and brought back to the laboratory immediately. Samples were washed by tape water for several times, then gently brushed and rinsed with distilled water and then dried at room temperature. Dried sample was pulverized using domestic blender and stored in air tight container for further use.

Extraction and phytochemical screening of seaweed

The active compounds in the powdered sample were extracted using organic solvents like acetone, ethanol, chloroform, petroleum ether, benzene and methanol, by percolation method. The powder was soaked in respective solvents for 48 hours and this procedure was repeated when the sample decolorized for thrice. Qualitative analysis of various extracts was subjected to identifying various bioactive constituents using standard procedures.[6]

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The dried seaweed powder sample was extracted with chloroform, using soxhlet extractor. The extract which is obtained is concentrated with rotary evaporator till dry powder was obtained. The final concentrated extract is analyses by using GC-MS.

GC–MS analysis was carried out on equipment Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II. Experimental conditions of GC-MS system were as follows: ZB 5-MS capillary standard non-polar column, dimension: 30Mts, ID: 0.25mm, Film thickness: 0.25µm. Flow rate of mobile phase (carrier gas: He) was set at 1.0ml/min. In the gas chromatography part, temperature programme (oven temperature) was 70°C raised to 260°C at 6°C/min and injection volume was 1µl. A sample dissolved in chloroform was run fully at a range of 50-650 m/z and the results were compared by using Wiley Spectral library search programme.

Identification of Components

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Antioxidant activities

Preparation and processing of the extracts and standard

10g of powdered sample was subjected to extract with chloroform using Soxhlet extractor for six hours and the extraction was repeated twice. The extracts were then concentrated to dryness under reduced pressure and controlled temperature (40-50^{o}C). The stock solutions were serially diluted with respective solvents to get lower concentrations (1000,750,500,250,100µg ml-1). Each concentration was prepared in triplicate. These were subjected to *in-vitro* assay of total antioxidant activity by the method of phosphomolybdate using α- tocopherol as the standard.[7] The total antioxidant capacity was expressed as µg g^{-1} equivalents of Vitamin E α-tocopherol by using the standard α-tocopherol graph. Nitric Oxide scavenging method can be determined by the use of the Griess Illosvoy reaction[8], Hydrogen peroxide by method of Dehpour *et al*., 2009[9], 1, 1-diphenyl-2-Picrylhydrazyl (DPPH) by Mensor *et al.*, 2001[10] and Hydroxyl radical scavenging methods by Chung *et al.*, 1997.[11] Vitamin C (Ascorbic acid) was used as a standard and was dissolved in respective solvent and diluted quantitatively to obtain a concentration of 100µg ml^{-1}.

$$\text{Scavenging activity (\%)} = [(A - B) / A] \times 100$$

Where A is the absorbance of control, B is the absorbance of the sample (extract/ ascorbic acid).

A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was calculated by linear regression analysis.

Calculation of IC50

IC50 value was calculated by using the formula to determine the regression equation Y= mx + c in MS Office excel version 2007.

Statistical Analysis

All experiments were carried out in triplicate (n=3). The results were presented as mean± SE standard error using SPSS statistics 17.0 software. The data were statistically analysed by one-way ANOVA and Tukey post-hoc test. The level of statistical significance was set at $p < 0.05$.

RESULT AND DISCUSSION

Phytochemical screening

Phytochemical constituents of a drug are helpful for identification purpose, when the drug is mixed together in compound drug formulation. Phytochemical analysis of *G. foliifera* is presented in Table – 1. Most of the bioactive compounds like alkaloids, catachin, coumarin, flavonoids, phenol, quinons, steroids, saponin, tannins, glycosides, amino acid, sugar and xanthoprotein were tested in various solvent extract. Whereas, compounds like catachin and xanthoprotein are not found. Most of the phytoconstituents are present in the chloroform extract of *G. foliifera*.

Table 1: Presence of phytoconstituents present in various extract of *G. folifera*

Phytoconstituents	Acetone	Ethanol	Chloroform	Petroleum ether	Benzene	Methanol
Alkaloids-Mayer's test	+	+	+	+	+	-
Catachin	-	-	-	-	-	-
Coumarin	+	+	+	+	+	+
Flavonoid	+	-	+	+	-	+
Phenol	+	+	+	-	-	+
Quinons	+	+	+	-	-	+
Saponin (Foam test)	-	+	+	+	+	-
Steroids	-	+	+	-	-	+
Tannins	+	+	+	-	-	+
Sugar-Benedict's test	-	-	+	+	+	+
Glycosides-Anthrone test	+	-	+	-	-	+
Amino acid	-	-	+	-	-	-
Xanthoprotein	-	-	-	-	-	-

(+) Present; (-) Absent

Gas Chromatography-Mass Spectrometry Analysis

In the present study the GC-MS analysis leads to the prediction of chemical constituent present in the chloroform extract of *G. foliifera.* Mass spectrometry is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds and to elucidate the structure and chemical properties of molecules. In figure 1 showed GC-MS chromatogram of chloroform extract of *G. foliifera*. The identification of the phytochemical compounds was confirmed based on their retention time (RT), molecular formula (MF), molecular weight (MW) and peak area in percentage (%) were tabulated in Table 2. GC-MS analysis of the phytochemicals present in Chloroform extracts of *G. foliifera* clearly showed the presence of thirty compounds were identify. Among these the compound Cholest-5-en-3-ol is a alcoholic compound and it may be employed as an antioxidant agents as confirmed by Dr. Duke's Phytochemical and Ethanobotanical Databases.[12] This identified phyto compound Cholest-5-en-3-ol use to antioxitant activity with the retention time (RT) 31.79 min and peak area 5.08% (molecular formula $C_{27}H_{46}O_2$, Molecular weight 386). In figure 2 and 3 represent the mass spectrum and structure of the identified compound of Cholest-5-en-3-ol (3a). This could be agreed by earlier studies, Selvaraju *et al.*, (2011)[13] reported that the brown seaweed *Sargassum wightii* possessed higher antioxidant content.

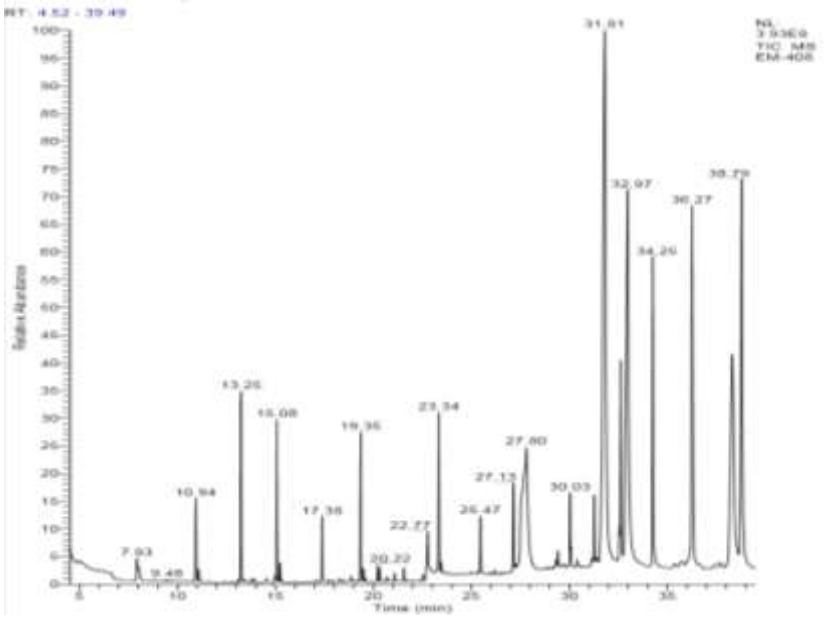

Figure 1: GC-MS chromatogram of chloroform extract of *Gracilaria foliifera*

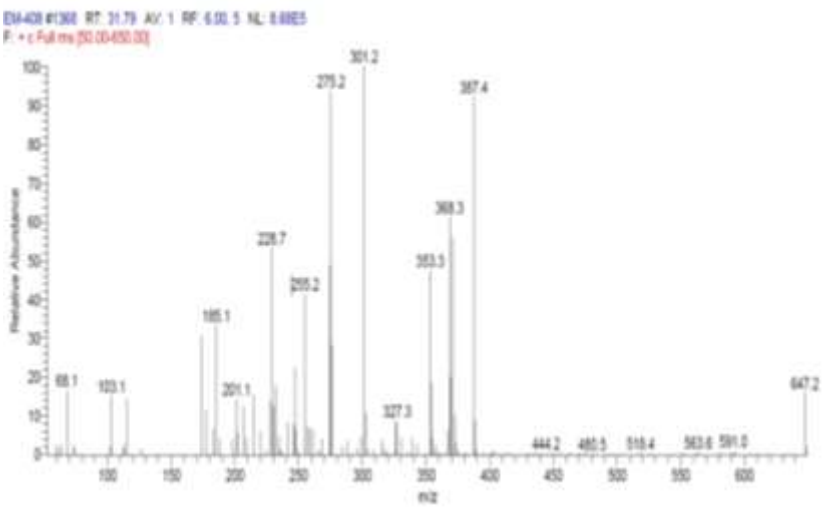

Figure: 2 Mass spectrum of identified compound of Cholest-5-en-3-ol (3a) (RT: 31.79).

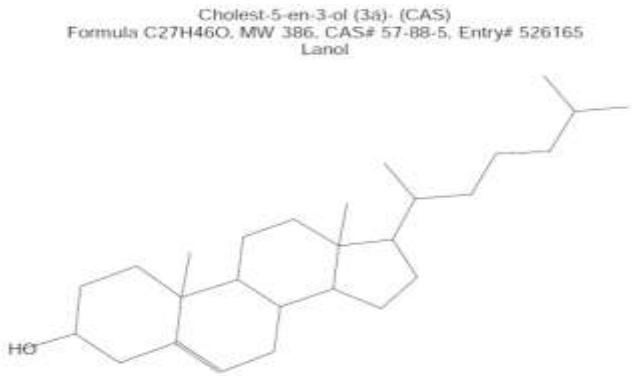

Figure: 3 Structure of identified compound of Cholest-5-en-3-ol (3a)

Table 2: GC-MS analysis showed phytochemical compounds of chloroform extract of red algae *Gracilaria foliifera*

S. No	RT	Name of the compound	Molecular Formula	Molecular Weight	Peak Area %
1	3.31	7-syn-Acetoxy-5-exo-hydroxy-3-exo methoxycarbonyl-bicy clo[2.2.1]heptane-2,6-carbolactone	$C_{12}H_{14}O_7$	270	77.60
2	3.78	Carbonocyanidic acid, ethyl ester	$C_4H_5NO_2$	99	0.20
3	5.00	2-[(N,N-Dipropylamino)methyl]piperidine	$C_{12}H_{26}N_2$	198	0.04
4	6.61	Methyl 1-amino-3-cyclopentene-1-carboxylate	$C_7H_{11}NO_2$	141	0.08
5	7.95	1-Decanol (CAS)	$C_{10}H_{22}O$	158	0.16
6	10.94	1-Hexadecene (CAS)	$C_{16}H_{32}$	224	0.30
7	13.25	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-	$C_{14}H_{22}O$	206	0.56
8	15.08	1-Hexadecanol (CAS)	$C_{16}H_{34}o$	242	0.44
9	17.38	HEPTADECANE	$C_{17}H_{36}$	240	0.17
10	19.35	E-15-Heptadecenal	$C_{17}H_{32}O$	252	0.42

11	20.25	Cyclopropyl methyl carbinol	$C_5H_{10}O$	86	0.07
12	21.56	PHENOL, 2,6-BIS(1,1-DIMETHYLETHYL)-4-METHYL	$C_{15}H_{24}O$	220	0.05
13	22.77	Hexadecanoic acid (CAS)	$C_{16}H_{32}O_2$	256	0.31
14	23.34	5- Eicosene	$C_{20}H_{40}$	280	0.56
15	25.47	2-Hexadecen-1-ol, 3,7,11,15- tetramethyl-, {R-[R*,R*-(E)]}	$C_{20}H_{40}O$	296	0.17
16	27.13	1-Heneicosyl formate	$C_{22}H_{44}O_2$	340	0.26
17	27.78	Pyrrolidine, 1-(1-oxo-5-(3-undecyloxiranyl)pentyl]-(CAS)	$C_{22}H_{41}NO_2$	351	1.94
18	29.42	3,4-Dihydro-8-hydroxy-9,12-dimethoxy-2,2,6,6-tetramethyl-2H,6H-	$C_{24}H_{26}O_6$	410	0.12
19	30.03	1-Heneicosyl formate	$C_{22}H_{44}O_2$	340	0.25
20	30.42	Hexadecanal (CAS)	$C_{16}H_{32}O$	240	0.03
21	31.28	Triacontane (CAS)	$C_{30}H_{62}$	422	0.22
22	31.79	Cholest-5-en-3-ol (3a)- (CAS)	$C_{27}H_{46}O$	386	5.08
23	32.62	Octacosane (CAS)	$C_{28}H_{58}$	394	0.64
24	32.97	Nonacosane (CAS)	$C_{29}H_{60}$	408	3.14
25	34.25	Octacosane (CAS)	$C_{28}H_{58}$	394	1.14
26	35.74	2-(N-ethylimino)propanenitrile	$C_5H_8N_2$	96	0.04
27	36.27	Octacosane (CAS)	$C_{28}H_{58}$	394	1.62
28	37.69	1-(Ethoxycarbonyl)-5-chloro-4-methoxy-3-nonylpentan-2-one	$C_{18}H_{33}ClO_4$	348	0.07
29	38.31	Nonacosane (CAS)	$C_{29}H_{60}$	408	2.26
30	38.79	Nonacosane (CAS)	$C_{29}H_{60}$	408	2.06

In-vitro Antioxidant activities

In-vitro antioxidant properties such as DPPH radical, H_2O_2, OH, NO radical scavenging activity and Total antioxidant activity of chloroform extract with concentration ranging from 100 to 1000µg/ml of of red algae *G. foliifera* was determined. The absorbance values, % inhibition, IC_{50} value, F- value and p- values of radical scavenging ability such as DPPH, H_2O_2, OH and NO at different concentration (100, 250, 500, 750, 1000 µg/ml*)* in chloroform extract of seaweed *G. foliifera* is shown in table 3.

Table 3: Different methods of radical scavenging activity of *G. foliifera* in chloroform extract compare with Standard Ascorbic acid

	Concentration (µg/ml)	Standard (Ascorbic acid)		Chloroform extract	
		Absorbance* (Absorbance λ 517 nm)	% Inhibition	Absorbance* (Absorbance λ 517 nm)	% Inhibition
DPPH Radical Scavenging activity	100	0.466±0.002^{d}	19.93	0.498±0.003^{d}	14.43
	250	0.363±0.002^{c}	37.62	0.405±0.013^{c}	30.41
	500	0.258±0.001^{b}	55.67	0.379±0.004^{c}	34.88
	750	0.156±0.004^{a}	73.19	0.291±0.008^{b}	50
	1000	0.153±0.004^{a}	73.71	0.236±0.005^{a}	59.45
IC_{50} Value *(µg/ml)*		486.99		779.36	
F- Value P- Value		3241.359 0.000		266.192 0.000	
H_2O_2 Radical scavenging activity	100	0.418±0.009^{a}	17.23	0.079±0.002^{a}	47.68
	250	0.566±0.025^{b}	25.03	0.122±0.009^{b}	54.48
	500	0.572±0.019^{b}	31.33	0.172±0.001^{c}	57.53
	750	0.670±0.009^{c}	48.54	0.234±0.006^{d}	61.26
	1000	0.552±0.016^{b}	55.30	0.316±0.007^{e}	61.51
IC_{50} Value *(µg/ml)*		855.38		75	
F- Value P- Value		43.318 0.000		105.898 0.000	
OH Radical Scavenging activity	100	0.254±0.009^{a}	45.38	0.263±0.005^{b}	43.44
	250	0.198±0.004^{a}	57.42	0.191±0.040 ^{ab}	58.92
	500	0.153±0.076^{a}	67.09	0.161±0.031^{ab}	65.38
	750	0.139±0.005^{a}	70.11	0.155±0.025^{a}	66.67
	1000	0.133±0.039^{a}	71.39	0.143±0.020^{a}	69.25
IC_{50} Value *(µg/ml)*		68.24		83.78	
F- Value P- Value		2.548 0.105		4.685 0.022	
Nitric Oxide Radical scavenging activity	100	0.251±0.071^{a}	45.67	0.265±0.021^{b}	42.64
	250	0.214±0.082^{a}	53.68	0.255±0.014^{b}	44.81
	500	0.186±0.090^{a}	59.74	0.243±0.011^{b}	47.40
	750	0.179±0.165^{a}	61.26	0.236±0.019^{b}	48.92
	1000	0.158±0.085^{a}	65.80	0.126±0.023^{a}	72.72
IC_{50} Value *(µg/ml)*		163.05		474.84	
F- Value P- Value		2.100 0.156		14.228 0.000	

**Values are expressed as mean ± SE (n=3) which, with different letters (within column), indicate significant difference (p<0.05). Post Hoc Test (Tukey HSD).*

Seaweed *G. foliifera* was assay for *in-vitro* total antioxidant activity of chloroform extracts equivalent with Vitamin E standard (α- tocopherol) and results were presented in Table-2. The standard regression curve present in fig.2 and the linear regression equation [y=0.0004x + 0.0807 (R^2= 0.9814)] obtained by Microsoft office excel.

Table 4: Total antioxidant activity of *G. foliifera* in chloroform extract equivalent of vitamin E standard *(a-Tocopherol)*

	Concentration *(µg/ml)*	Standard	Chloroform extract	
		a-Tocopherol (Vitamin E) Absorbance (λ 695 nm)*	Absorbance*	*µg*/ml *equivalent of vitamin E standard (a-Tocopherol)*
Total antioxidant activity	100	0.105±0.001[a]	0.055±0.0004[a]	137.5
	250	0.177±0.006[b]	0.064±0.005[ab]	160
	500	0.259±0.010[c]	0.074±0.009[ab]	185
	750	0.378±0.015[d]	0.093±0.012[bc]	232.5
	1000	0.421±0.011[e]	0.120±0.008[c]	300
F- Value		272.889	15.799	
P- Value		0.000	0.000	

**Values are expressed as mean ± SE (n=3) which, with different letters (within column), indicate significant difference (p<0.05). Post Hoc Test (Tukey HSD)*

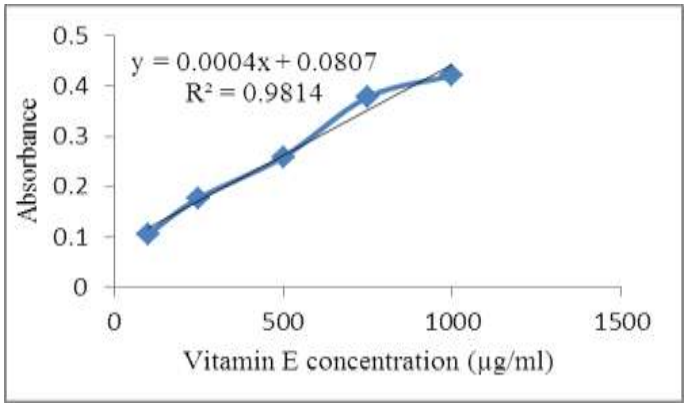

Fig 4: Standard curve for total antioxidant activity assay using α-tocopherol (100-1000*µg/ml)*

The results of this study shows that the *in-vitro* antioxidant activity by DPPH, H_2O_2, OH and NO radical scavenging activity were expressed in terms of % inhibition and IC_{50} value with respect to chloroform extract of *G. foliifera* have significance result to compared with standard. Naturally seaweeds contain large amounts of antioxidant compounds which control the free radical formation from metabolic reaction. Natural antioxidants found in algae play an important role against various diseases and ageing processes by protecting the cell from oxidative damage.[14] In earlier, many workers report seaweeds are considered to be a rich source of antioxidants.[15-18] The results have showed that the chloroform extracts of *G. foliifera* of marine red algae exhibit antioxidant potential and they are sources of natural antioxidant compounds.

CONCLUSION

The antioxidant activity of chloroform extract of marine red algae *G. foliifera* was determined by using DPPH, H_2O_2, OH and NO testing. Antioxidant activity measured using DPPH is reasonable with other potential to reduce free radicals compare with other test. The presences of phytoconstituents like flavonoid, phenols, quinons etc have been recognized as having the potential to reduce the risk of chronic diseases. The characteristic of antioxidant activity of *G. foliifera* is an agar yielding agarophyte should be used as neutraceutical application.

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REFERENCE

1. Mayer AMS, Rodríguez AD, erlinck RGS and Fusetani N. Marine pharmacology in 2007-8: Marine compounds with antibacterial, anticoagulant, antifungal, antiinflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action. Comp Biochem Phys C., 2011; 153: 191-222.
2. Pires KMS, Alencar DB, Sousa MB, Sampaio AH and Saker-sampaio S. Teores de α- e β-caroteno em macroalgas marinhas desidratadas. Rev Cienc Agron, 2008; 39: 257-262.
3. Pires-cavalcante KMS, Alencar DB, Sousa MB, Sampaio AH and Saker-sampaio S. Seasonal changes of α-tocopherol in Green marine algae (*Caulerpa* genus). J Food Sci., 2011; 76: 775-781.
4. Sousa MB, Pires KMS, Alencar DB, Sampaio AH and Saker-sampaio S. α-, β-caroteno e α-tocoferol em algas marinhas in natura. Ciencia Tecnol Aliment, 2008; 28: 953-958.
5. Wu XJ and Hansen C. Antioxidant capacity, phenolic content and polysaccharide content of Lentinus edodes grown in whey permeate-based submerged culture. J Food Sci., 2008; 73(1): M1–8.
6. Harborne JB. Phytochemical methods. *Chapman & Hall, New York*, 1973; 288.
7. Prieto P, Pineda M & Aguilar M. "Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E." *Anal. Biochem*, 1999; 269: 337.
8. Garrat DC. The quantitative analysis of drugs. Chapman and Hall ltd, Japan. *Biochem. Anal. Chem.*, 1964; 3: 456.

9. Dehpour AA, Ebrahimzadeh MA, Nabavi SF & Nabavi SM. Antioxidant activity of the methanol extract of *Ferula assafoetida* and its essential oil composition. *Grasas y Aceites*, 2009; 60: 405.
10. Mensor LI, Menezes FS, Leitao G, Reis AS, dos Santos T, Coube CS & Leitao SG. Screening of Brazillian plant extracts for antioxidant activity by the use of DPPH free radical method, *Phytotherapy Research*, 2001; 15: 127.
11. Chung S K, Osawa T & Kawakishi S. Hydroxyl radical scavenging effects of spices and scavengers from brown mustard (*Brassica nigra*), *Biosci. Biotechnol. Biochem*, 1997; 61: 118.
12. Dukes. Phytochemical and Ethnobotanical Databases. Phytochemical and Ethnobotanical Databases. www.ars-gov/cgi-bin/duke/. 2013.
13. Selvaraju M, Shanmugam U, Muthuvel A & Thangavel B. *In vitro* antioxidant properties and FTIR analysis of two seaweeds of Gulf of Mannar, *Asian Pac J Trop Biomed*, 2001; 1: S66.
14. Karawita R, Senevirathne M, Athukorala Y, Affan A, Lee YJ, Kim SK, Lee JB & Jeon YJ. Protective effect of enzymatic extracts from microalgae against DNA damage induced by H2O2. *Marine Biotechnol*, 2007; 9: 479.
15. Cahyana AH, Shuto Y, Kinoshita Y. Pyropheophytin a as an antioxidative substance from the marine alga arame (Eisenia bicyclis). Biosci. Biotechnol. Biochem, 1992; 56: 1533-1535.
16. Devi GK, Maniyannan K, Thirumaran G, Rajathi FAA, Anantharaman P. In vitro antioxidant activities of selected seaweeds from Southeast coast of India. Asian Pac. J. Trop. Med., 2011; 4: 205-211.
17. Kelman D, Posner EK, McDermid KJ, Tabandera NK, Wright PR, Wright AD. Antioxidant Activity of Hawaiian Marine Algae. Mar. Drugs, 2012; 10(2): 403-416.
18. Kim C, Lee IK, Cho GY, Oh KH, Lim YW, Yun BS. Sargassumol, a novel antioxidant from the brown alga Sargassum micracanthum. J. Antibiot, 2012; 65: 87-89.