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FREE RADICAL SCAVENGING AND INHIBITION OF PROTEIN DENATURATION CAPABILITY OF CHLOROFORM EXTRACT OF MARINE ALGA *GRACILARIA CORTICATA* (J. AGARDH).

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ABSTRACT

Seaweeds are one of the richest and essential resources in the marine ecosystem, which are the major sources for various biologically active components and also have been used as a source of food and medicine. In this present study we assessed the various extracts of *Gracilaria corticata*, a red algae, for anti-oxidant and anti-inflammatory potency if any. Successive extracts of *Gracilaria corticata* were prepared and subjected to anti-oxidant activity. Among the extracts, chloroform extract of *Gracilaria corticata* were showed good anti-oxidant activity when compared to standard. Hence it was analyzed by GC-MS technique for the identification of constituents responsible for the activity and it was also tested for anti-inflammatory property by protein denaturation technique. Among each concentration, chloroform extract excibited respectable activity with utmost inhibition og about 81.08% at a concentration for which the percentage inhibition is 98.3%. The whole study shown that *Gracilaria corticata* has appreciable free radical scavenging activity and significant anti-inflammatory property with a scope of further bioassay guided screening of the active components.

KEYWORDS: *Gracilaria corticata,* Seaweed, Free radical Scavenging, protein denaturation, GC-MS.

INTRODUCTION

Seaweeds are macroscopic, marine, saltwater-dwelling a primitive type of plants which form an important component of the marine living resources.[1] Marine algae contain more than 60 trace elements in a concentration much higher than in terrestrial plants and have various pharmacological activities.[2] At one point of time, around the year 1970, Ryther and collaborated have assessed various species of Red, Green and Brown seaweeds for the enhanced growth rates as well as its yields at drying and powdering.[3] They exclusively focused on the Genus Gracilaria because of its yield after drying and also produces commercially valuable extracts.[4] *Gracilaria corticata* genus (Gracilariales, Rodophyta) is a macro algae group with more than 300 species of which 160 have been accepted taxonomically.[5,6] Though the huge species of the available *Garcilaria,* only 19 of them were tested chemically for the presence of various pharmacological activity. These are usually red algae with a 3-phase cycle and can be found in tropical and subtropical seas. Lipids are abundant in this genus being mainly prostaglandins[7], steroids, such as cholesterol and clinoasterol are present in this respectively.[8] *Gracilaria corticata* used as antibiotic, anti-HIV, anti-inflammatory[9], anti coagulant, anti-ulcer[10] wound healing, hepatoprotective[11] and anti tumor agents[12] So, the seaweed *Gracilaria corticata* was selected based on preliminary screening was evaluated for its pharmacological activity such as anti-oxidant and anti-inflammatory.

MATERIALS AND METHODS

Collection of seaweed

Gracilaria corticata were collected from mandapam station which is located in the Gulf of Mannar region, Rameswaram, India. It was authenticated by Dr.Ganesan, Senior Scientist, Central Salt and Marine chemical Research Institute (CDIR) (Marine Algal Research Station) Mandapam, Ramanathapuram District, Tamilnadu. Algae samples were collected by handpicking. They were washed and made free from extraneous matter such as epiphytes, sand particles, pebbles and shells brought to laboratory in plastic bags. The samples were then, thoroughly washed with fresh water. They were shade dried, powdered, sieved and preserved.

Preparation of extract

About 500g of dried algal (*G.corticata*) powder was extracted with Hexane by Hot maceration technique for 8 hrs; Extract was filtered off using sterile filter paper (Whatmann No:1) into a sterile conical flask and the collected extract was evaporated to make the volume $1/4^{th}$ of the original volume, and stored in a air tight

bottles for further studies and the marc was dried and again extracted with chloroform using the same procedure as above and collected, dried and stored for further studies. Similarly the procedure was repeated for Ethyl acetate and Ethanol and the extract was stored in an air tight container for further studies.

Phytochemical screening

The different extracts were tested for steroids, triterpenoids, reducing sugars, phenolic compounds, saponins, xanthoproteins, tannins, flavanoids, proteins, glycosides and anthroquinones. Phytochemical screening of the extracts was carried out according to the standard methods.[13,14]

Table 1: Preliminary Phytochemical analysis of various extracts of Gracilaria corticata

NAME OF TEST	HEXANE	CHLOROFORM	ETHYL ACETATE	ETHANOL
Terpenoids	-	-	-	+
Flavonoids	-	+	-	+
Steroids	+	+	+	-
Anthraquinones	-	-	-	-
Glycosides	-	-	+	-
Quinones	-	-	-	-
Phenols	+	+	+	-
Tannins	+	-	-	-
Saponins	+	+	-	-
Proteins and amino acids	-	-	-	-
Carbohydrate	-	+	-	+
Alkaloids	+	-	-	-

Anti-oxidant activity

Nitric Oxide Radical Scavenging Assay

In this assay sodium nitroprusside in aqueous solution spontaneously generate nitric oxide, which interacts with oxygen to produce nitrite ions and the same can be estimated by using Griess reagent. Griess reagent contain 0.1% w/v naphthyl ethylene diamine scavengers of nitric oxide helps to reduce the production of Nitrite ions by competing with Oxygen.

3 ml of Different Seaweed extracts with the concentration ranging from 0.5-2.5 ml was incubated at 25^{o}C for 150 mins along with Sodium nitroprusside (5M) in phosphate-buffered saline (1xPBS pH 7.4). It is then combined with Griess reagent and the as a result of combination of Sulphanilamide with Naphthyl Ethylenediamine diazotization of nitride happened and the absorbance of the chromophore was read at 546nm. Postitive control used was Asxcorbic acid and percentage scavenging of nitric acid was obtained by means of calculation using the formula:

NO Scavenging (%) = [(absorbance of control - absorbance of test sample) / absorbance of the control] x 100

The results were tabulated in Table 2 and Figure 1.

Table: 2 Percentage inhibition of various extracts of *G.corticata* at 546nm.

Concentration of the extract (µg/ml)	Percentage inhibition (%)				
	Hexane	Chloroform	Ethyl Acetate	Ethanol	Ascorbic acid
0.25	42.80	50.26	52.32	42.11	48.32
0.50	51.45	53.12	50.41	50.26	57.16
1.0	62.34	68.18	59.46	58.39	69.7
1.5	69.23	71.92	62.70	63.66	74.82
2.0	79.36	80.16	70.42	73.90	81.80
2.5	73.82	81.32	73.24	74.20	84.19

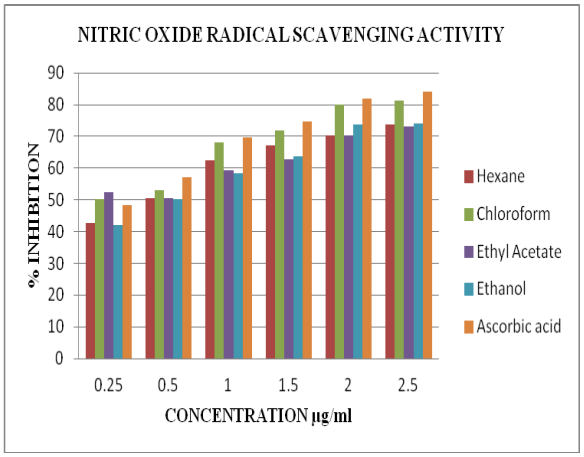

Figure1: Nitric oxide scavenging effect of successive seaweed extract of *G.corticata*

Reducing Power Assay

As per the procedure mentioned by the literature.[15] Reducing Power assay procedure was carried out and thus helps us in measuring the total anti-oxidant capacity of the sample in turn evaluating the redox potential of the compounds.

1ml of various solvents soluble extracts of selected seaweed at various concentration after mixing with 2.5 ml of Phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of Potassium Ferric Cyanide (1%) was kept at 50°C in water bath for 20 mins. After the incubation period, along with this reaction mixture, 2.5 ml of Trichloror acetic acid was added drop wise and centrifuged by fixing at 650 rpm for about 10 mins. From the supernatant layer of about 2.5ml, 2.5ml of distilled water and 0.5ml of Ferric Chloride (0.1%) was added and absorbance was measured at 700 nm. Increased absorbance indicates increased reducing power. The percentage inhibition was calculated using the formula below and the results were shown in Table 3 and Figure 2.

Percentage increase in reducing power = (absorbance of test / absorbance of blank) – 1x100

Table3: Percentage inhibition of various extracts of *G.corticata* at 700nm.

Concentration of the extract (µg/ml)	Percentage inhibition				
	Hexane	Chloroform	Ethyl Acetate	Ethanol	Ascorbic acid
62.5	44.6	47.3	40.3	39.6	48.6
125	49.2	54.3	44.2	48.7	57.2
250	62.3	69.9	60.6	63.3	71.6
500	70.6	78.3	66.3	74.8	79.4
1000	78.3	80.6	71.2	75.6	83.2
2000	82.6	87.5	80.3	79.7	89.7

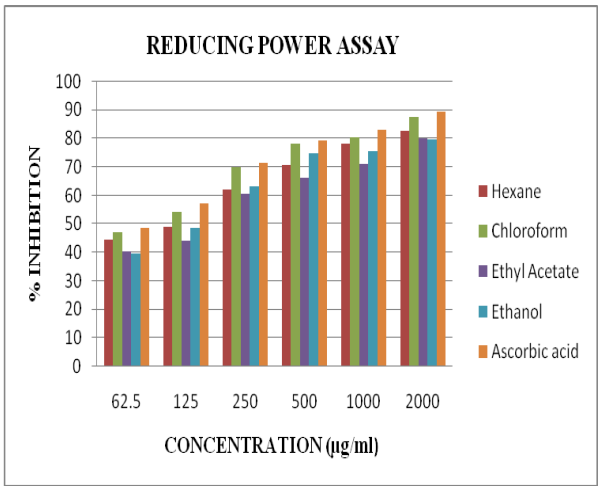

Figure 2: Reducing power activity of successive seaweed extracts of *G.corticata.*

Gas Chromatography–Mass Spectroscopic examination

Since the chloroform extract of *Gracilaria corticata* has shown good anti-oxidant activity when compared to the standard Ascorbic acid, it was subjected to GC-MS analysis. GC-Ms is one of the powerful technology used v\wide range of applications in various fields, especially pharmaceutical in identification of active constituents from natural sources and the results obtained has high sensitivity and specificity. This technique helps us in identification of compounds based on the molecular weight of the constituents present. Gas chromatography (GC) helps in the ideal separation of constituents followed by the attachment with mass spectroscopy (MS) determines the molecular weight of the isolated components, thus helps in both Qualitative and Quantitative analysis of Volatile and semi volatile compounds.

The additional defined information in qualitative examination can be attained by gas chromatography together with mass spectroscopy (GC-MS). The GC-MS analysis of *Gracilaria corticata* seaweed extract within absolute alcohol was achieved using clarus 500 Perkin Elmer Gas Chromatography along with Elite-5 capillary column(5% phenyl 95% dimethyl polysiloxane) (30 nm x 0.25 ID x 0.25µmdf). Mass detector of Turbo mass gold company was functioning in EI mode. Carrier gas used was Helium at a run time of 1 ml/min. The injector was controlled at 250^0C and the oven temperature was automated as follows; 50^0C at 8^oC/min to 200^oC (5 min) at 7^oC/min to 290^oC (10 min) at 110^oC (2 min) to 200^oC at 10^oC/min to 280^oC (9 min) at 5^oC/min. Total operation

time in 36 mins. The effective volatile constituents present in the extract was achieved using GC-MS and the interpretation on mass spectrum of GC-MS was concluded using the Database of Indian Institute of Crop Processing Technology (IICPT) having more than 75,000 patterns. The chloroform extract of *Gracilaria corticata* seaweed was liquefied on chloroform and passed through a filter with polymeric solid phase extraction (SPE) column and investigated in GC-MS for different components. The results of the components identified in chloroform extract were displayed in Table 4 and Figure 3.

Table 4: Components identified in the chloroform extract of *G.coticata*

S.NO	RT	NAME OF THE COMPOUND	MOLECULAR FORMULA	MOLECULAR WEIGHT	PEAK AREA%
1	7.35	Octane,3,4,5,6-tetramethyl-	$C_{12}H_{26}$	170	0.03
2	7.71	Phenol,2,4-bis(1,1- dimethyl ethyl)-	$C_{14}H_{22}O$	206	0.17
3	8.66	3,4-dihydroxy-5-aminopyridazine	$C_4H_5N_3O_2$	127	0.17
4	9.78	Decane,2,9-dimethyl-	$C_{12}H_{26}$	170	0.10
5	10.93	3,3-dimethyl-hepta-4,5-dien-2-ol	$C_9H_{16}O$	140	0.87
6	11.35	2-pentadecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	268	1.42
7	11.63	1,2- benzenedicarboxylic acid,bis(2-methyl propyl) ester	$C_{16}H_{22}O_4$	278	1.46
8	12.16	1,2-benzenedicarboxylic acid,butyl 2-ethyl hexyl ester	$C_{20}H_{30}O_4$	334	9.61
9	12.73	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	28.37
10	12.92	1,2 –benzenedicarboxylic acid,butyl cyclohexyl ester	$C_{18}H_{24}O_4$	304	22.34
11	14.57	L-serine ,O-(phenyl methyl)-	$C_{10}H_{13}NO_3$	195	8.64
12	15.84	5,5,10,10-tetrachlorotricyclo[7.1.0.0(4,6)]decane	$C_{10}H_{12}Cl_4$	272	6.21
13	17.69	10-methyl-8-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268	6.07
14	20.34	1,2-benzenedicarboxylic acid,diisooctyl ester	$C_{24}H_{38}O_4$	390	1.77
15	24.86	Cholesta-4,6-dien-3-ol,(3α)-	$C_{27}H_{44}O$	384	2.81
16	27.60	Stigmasterol	$C_{29}H_{48}O$	412	0.42
17	28.18	Cholesterol	$C_{27}H_{46}O$	386	9.54

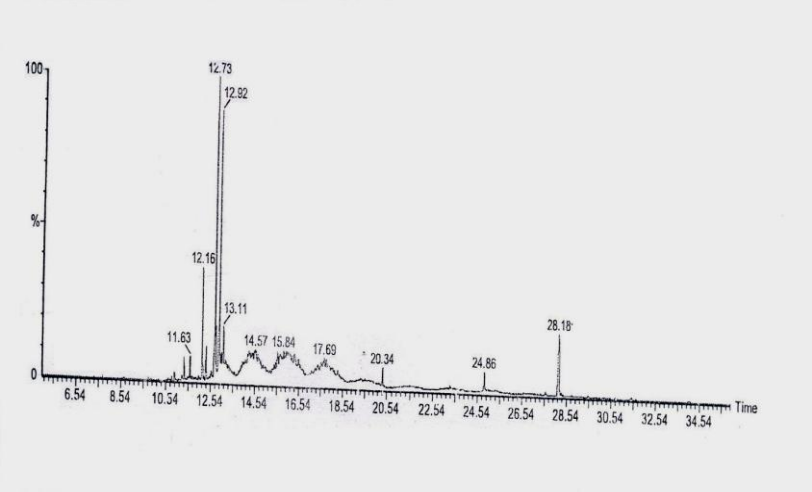

Figure3: GC-MS spectrum of chloroform extract of *G.corticata*.

Anti-inflammatory activity

Protein Denaturation Technique

In the present study the protein denaturation bioassay was selected for *in-vitro* assessment of anti-inflammatory property of the best extract, based on *in-vitro* anti-oxidant assay of *Gracilaria corticata* seaweed. Denaturation of tissue proteins is one of the main causes of inflammatory and arthritic disease. Production of auto antigens in certain inflammatory condition may be due to denaturation of proteins *in-vivo*. Agents that can prevent protein denaturation therefore, would be worthwhile for ant-inflammatory drug development.

The test solution (0.5 ml) consists of 0.45 ml of Bovine Serum Albumin (5% w/v aqueous solution) and 0.05 ml of test solution. Test control solution (0.5ml) consists of

0.45 ml of Bovine Serum Albumin (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control (0.5 ml) consists of 0.45 ml of distilled water and 0.05 ml of test solution. Standard solution consists of 0.45 ml of Bovine Serum Albumin (5% w/v aqueous solution) and 0.05 ml of Diclofenac sodium. Various concentrations (50, 100, 250, 500, 1000, 2000 µg/ml) of algal extracts (test solution) and Diclofenac sodium (standard solution) were taken respectively. All the above solutions were incubated at 37^{o}C for 3 mins. It is then cooled and Phosphate buffer of about 2.5 ml was added to it and absorbance was measure at 416 nm. Standard used was Diclofenac sodium. The percentage inhibition of protein denaturation was measured by calculation using the formula:

Percentage inhibition = [100 - (optical density of test solution – optical density of product control)/ (optical density of test control)]

The results of the component were displayed in Table 5 and Figure 4.

Table 5: Percentage inhibition of chloroform extract *of G.corticata*

S.NO	CONCENTRATION (µg/ml)	% INHIBITION	
		DICLOFENAC SODIUM	*Gracilaria corticata*
1	31.2	94.1	34.26
2	62.5	94.3	40.28
3	125	98.10	57.23
4	250	98.64	61.66
5	500	98.27	70.14
6	1000	98.3	81.08

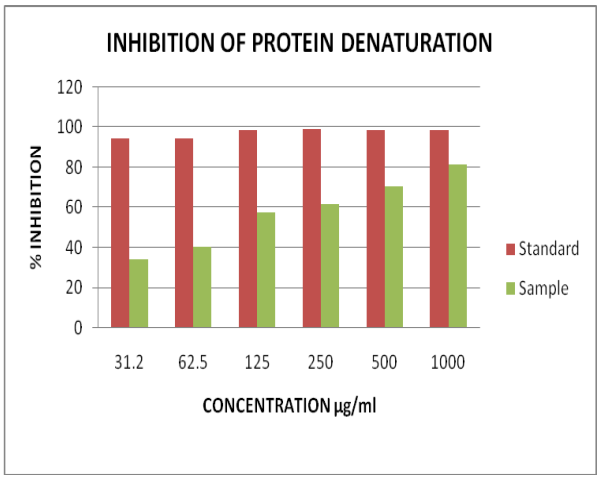

Figure 4: Percentage inhibition of Protein denaturation by chloroform extract of *G.corticata*.

RESULTS AND DISCUSSION

PRELIMINARY PHYTOCHEMICAL SCREENING

Thirteen different components including Steroids, Saponins, Anthraquinones, Carbohydrates, Carboxylic acids, Coumarins, Proteins, Flavonoids, etc were tested for their presence or absence in all the prepared extracts of the selected seaweed. The tabular column consisting of the constituents answered positively for all four extracts were displayed in Table No: 1.

Among the four different extracts, (Hexane, Chloroform, Ethyl acetate and methanol) Hexane and Chloroform extracts showed the presence of maximum number (5 each) of compounds. Steroids and Phenols showed the maximum presence in three different extracts of *Gracilaria corticata* followed by alkaloid, carbohydrate and tannin in 2 extracts. The behavior of the drug powder with different chemical reagent will also be helpful in characterization of the crude drug.

ANTI-OXIDANT ACTIVITY

a. Nitric Oxide Radical Scavenging Assay

The scavenging of nitric oxide was found to be amplifying in dose dependent manner. Maximum absorbance was observed in the Chloroform Extracts of *Gracilaria corticata*, when compared to standard Ascorbic acid. At the highest concentration of 2.5µg/ml the percentage inhibition of Chloroform extract was found to be 81.32% when compared to standard Ascorbic acid used which is 84.19%. The results were exposed in Table 2 and Graphically in figure 1.

b. Reducing Power Assay

Similarly all four extracts were tested by reducing power assay method. Again among various Extracts, the highest percentage inhibition of Chloroform extract of *Gracilaria corticata* was about 87.5% at concentration of 2000µg/ml in contrast to standard which was about 89.7% at same concentration was observed. The results were exposed in Table 3 and graphically in Figure 2. Hence the chloroform extract alone was submitted to GC-MS analysis

GC-MS ANALYSIS

Since the Chloroform extract have found to possess good anti-oxidant activity as compared to standard Ascorbic acid it was subjected GC-MS analysis to unveil the chloroform soluble constituents that exists within and responsible for the activity. The GC-MS spectrum revealed certain compounds of excellence were found to exist in it which may be reason for the enhanced percentage of anti-oxidant activity when compared to the standard Ascorbic acid. Moreover it was found in the previous literatures that the compounds which are

excellence in anti-oxidant capacity, may also have anti-inflammatory property. Hence, Chloroform extract of *Gracilaria corticata* were tested for its anti-inflammatory potency using Protein Denaturation assay. The results were exposed in Table 4 and graphically in Figure 3.

ANTI-INFLAMMATORY ACTIVITY

Based on in-vitro anti-oxidant assay results and GC-MS analysis, Chloroform extracts of *Gracilaria corticata* were tested for its anti-inflammatory potency by Protein Denaturation assay, using Diclofenac Sodium as standard. Among each concentration, Chloroform extracts of *Gracilaria corticata* exhibited respectable activity with utmost inhibition of about 81.08% at a concentration of 1000µg/ml when parallel to standard Diclofenac sodium at the same concentration for which the percentage inhibition is 98.3%. The results are presented in Table 5 and Figure 4.

CONCLUSION

Taking into consideration of above discussed results when compared to other successive extracts, chloroform soluble constituents participate in major pertaining to its anti-oxidant and anti-inflammatory property of the seaweed *Gracilaria corticata*. Hence further work is necessary to identify and to isolate the specific constituents either as single constituent or in combination if any synergistic action is noted and to elucidate the mode of action of these drugs or in combinations are the need of the hour and we are already in process and progress regarding this.

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