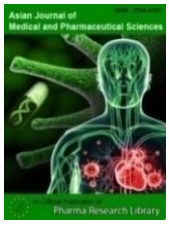

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Phytochemical Analysis and Pharmacological Evaluation of Phlorotannins Isolated from Marine Brown Algae Sargassum Wightii

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A B S T R A C T
Oxidative stress has been implicated in the connection of diseases such as diabetes, cardiovascular diseases, liver cirrhosis nephrotoxicity, cancer, and aging, etc. Antioxidant substrates from plants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation, and by other mechanisms. Natural antioxidant defense systems protect biomolecules against ROS/RNS induced damage, and re-establish or maintain redox homeostasis. Sea weeds are known to produce a variety of compounds and several of them have been shown to possess biological activity of potential medicinal values. For centuries, several seaweeds have been utilized traditionally as food supplements for various medical conditions. High-performance liquid chromatographic analysis was performed for ethyl acetate fraction (E4) of S. Wightii using mobile phase methanol: water ratio of 85:15 flow rate 1ml/min wavelength at 254nm against reference standard phloroglucinol. For the E4 fraction a peak obtained at retention time 2.704 min and for standard phloroglucinol at 2.762 min. tailing factor of the sample is 1.430 whereas phloroglucinol 1.078. The major causes for oxidative damage include Reactive Oxygen Species (ROS). ROS are produced by all aerobic organisms and can easily react with most biological molecules including protein, lipids, lipoproteins, DNA, RNA and enzymes leading to cell or tissue injury. Experimental tumors have great importance in modeling, and HeLa cell line is one of the old and commonest cervical cancer cell lines. In the present study HeLacell lines were used to screen the anti-cancer or cytotoxicity potential of the extracts of Sargassum wightii, using preliminary screening technique. The cytotoxicity increased with increase in concentration of phlorotannin rich extract.
Keywords: Oxidative stress, ROS/RNS, HeLacell, JNC 8 guidelines

A R T I C L E I N F O

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C o n t e n t s

1. Introduction

Algae are considered a big group of autotrophic organisms, ranging from unicellular to multicellular forms. These organisms can be found in both marine and fresh waters, and are divided according to their size into microalgae and macroalgae/ seaweeds. Microalgae constitute a polyphyletic group of prokaryotic and eukaryotic microscopic organisms with a simple cellular structure.

Phlorotannins: Phlorotannins, the dominant polyphenolic secondary metabolites found only in brown algae (Phaeophyta). There are fundamental differences in the chemical structures of polyphenols in both terrestrial & marine plants. Where as in terrestrial plants polyphenols are derived from is Gallic acid & Ellagic acids.

Figure: 1 *Sargassum wightii*

Classification:

Empire : Eukaryote
Kingdom : Chromista
Phylum : Ochrophyta
Class : Phaeophyceae
Subclass : Fucophycidae
Order : Fucales
Family : Sargassaceae
Genus : Sargassum
Species : wightii

Chemical Constituents:

Terpenoids, flavonoids, sterols, sulfated polysaccharides, polyphenols, sargaquinoic acids, sargachromenol, pheophytine .

Claimed uses: Analgesic, anti-inflammatory, antioxidant, neuroprotective, antimicrobial, antitumor, fibrinolytic, immune modulatory, anticoagulant, hepatoprotective, antiviral activity etc (Subhashyende et al., 2021).

2. Materials and Methods

Chemicals

Chloroform, n-Hexane, Methanol (HPLC grade), deionised water, ethyl acetate are of analytical grade were used. 1,1-Dipheny1-2picryl hydrazyl (DPPH), 2,2' –Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), Bovine serum albumin, were obtained from Sigma Aldrich Co., St. Louis, USA. Phosphate Buffer Saline from Gibco-BRL. Rutin from Acros Organics., New Jersey, USA. Naphthyl Ethylene Diamine Dihydrochloride (NEDD) from Roch – Light Ltd., Suffolk, UK. Ascorbic acid from SD Fine Chemicals Ltd., Mumbai, India. Sodium nitroprusside, Dimethyl sulphoxide, Potassium chloride and Sodium chloride from Ranbaxy Laboratories Ltd., Mohali, India. Sulphanilic acids, Sodium bicarbonate, Disodium hydrogen phosphate from E-Merck (India) Ltd., Mumbai, India. All chemicals used were of analytical grade.

Instruments

Analytical UV-Visible Spectrophotometer (Analytical Technologies Ltd, Model no: AUV 2060), Electronic Balance (Shimadzu, Model no: DS-852 J), Homogenizer (Ever Shine, Model no: 607), Centrifuge (Remi, Model no: KKLO-9013). Semi Autoanalyser (Mispa excel, version: 1.4e), HPLC Simazdu, Rotary flash evaporator

Pharmacognostic Evaluation:

Ash values determination:

Ash values are helpful in determining the quality and purity of crude drugs in powder form. Ash values such as total ash, water soluble ash and sulphated ash values were determined.

Total Ash value:

About 2 gm of accurately weighed air dried powdered drug was taken in a tare silica crucible and incinerated at a temperature not exceeding 450□C until free from carbon. Cooled and weighed, repeated for constant weight. Then the percentage of total ash was calculated with reference to the air-dried drug 35.

Water soluble Ash value:

The total ash obtained was boiled with 25 ml of water for 5 minutes. The insoluble matter was collected on an ash-less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450□C. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried crude drug.

Tests for carbohydrates:

Molish's test (General test): To 2 –3 ml test solution (T.S.) and a few drops of α-napthol solution in alcohol was added. The mixture was shaken and concentrated KOH was added from the sides of the test tube. A violet ring was observed at the junction of two liquids.

For reducing Sugars:

Fehling's test: 1 ml Fehling's A and 1ml Fehling's B were mixed and boiled for 1 minute. Added equal volume of TS was added and heated in boiling water bath for 5 – 10 min, first it emitted a yellow and then brick red precipitate.

Benedict's test: Equal volume of Benedict's reagent and T.S. were mixed in a test tube. Heated on a boiling water bath for 5 min. solution appears green, yellow or red depending on amount of reducing sugar present in the test solution 38.

Test for Monosaccharides:

Barfoed's test: Equal volumes of Barfoed's reagent and T.S. were added. Heated for 1–2 mins, in boiling water and cooled, the red precipitate was formed.

Tests for Hexose Sugars:

Cobalt–Chloride test: 3 ml of test solution was mixed with 2 ml cobalt chloride, boiled and cooled. Few drops of FeCl3 on NaOH solution were added. The solution showed greenish blue (glucose), purplish (fructose) or upper layer greenish blue and lower layer purplish (Mixture of glucose and fructose).

Tests for non –reducing sugars: T.S. does not give response to Fehling's and Benedict's test.

Tannic acid test for starch:

With 20% tannic acid, test solution white precipitate formed.

Tests for Proteins:

Biuret test (General test):

To 2 -3 ml T.S. 4% NaOH and a few drops of 1% CuSO4 solution was added. A violet or pink colour was observed. Millon's test (for proteins): To 5 ml millon's reagent, 3 ml T.S. was added. It formed white precipitate. When warmed the precipitate dissolves giving brick red or precipitate red colour. Xanthoprotein test (For protein containg tyrosine or tryptophan): 3 ml test solution was mixed with 1ml concentrated H2SO4. White precipitate was formed.

Test for protein containing sulphar:
5ml test solution was mixed with 2 ml 40% NaOH and 2 drops 10% of the lead acetate solution. When boiled the solution turned black or brownish due to PbS formation.

Precipitation test:
The test solution gave white colloidal precipitate with following reagents:
Absolute alcohol
5% HgCl2 solution
5% CaSO4 solution
5% Lead acetate
5% Ammonium sulphate

Tests for steroid:

Salkowski reaction: To 2 ml of T.S. 2 ml chloroform and 2 ml conc. H2SO4 was added. It was shaken well until, chloroform layer appeared red and acid layer showed greenish yellow fluorescence.

Liebermann – Burchard reaction: 2 ml T.S. with was mixed with chloroform. 1- 2 ml acetic anhydride and 2 drops conc. H2SO4 were added from the side of the test tube. First red, then blue and finally green colour was observed.

Liebermann's reaction: 3 ml T.S. was mixed with 3 ml acetic anhydride. The mixture was heated and cooled. A few drops concentrated H2SO4 were added and observed for blue colour.

Tests for Amino acids:
Ninhydrin test (General test): 3 ml T.S. and 3 drops 5% Ninhydrin solution were heated in boiling water bath for 10 minutes. A bluish purple colour was observed.
Test for Tyrosine: 3 ml T.S. was heated and 3 drops millon's reagent were added to it. The solution turned dark red colour was observed.
Test for Tryptophan:
To 3 ml T.S. A few drops glycoxalic acid and concentrated H2SO4 were added. Observed for reddish violet ring at junction of the 2 layers.

Tests for Glycosides:

Tests for Cardiac Glycosides:

Baljet's test: A T.S. changed from for yellow to orange colour with sodium picrate.

Legal's test (for Cardenolides):
To aqueous or alcoholic test solution, 1ml pyridine and 1 ml sodium nitroprusside were added the colour turned pink and changed red.

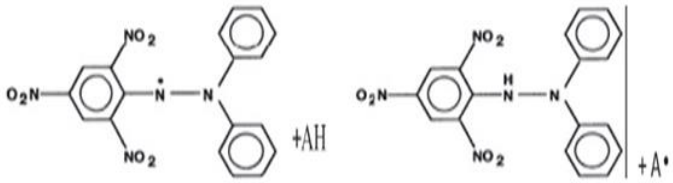
Test for deoxy sugars (Keller Killiani test):
To 2 ml T.S. glacial acetic acid, one drop of 5% FeCl3 and conc.H2SO4 were added. It gave rise to reddish brown colour at the junction of the 2 liquids and the upper layers appeared bluish green.

Libermann's test (for Bufadienolides):
3 ml T.S. was mixed with 3 ml acetic anhydride. The mixture was heated and cooled. A few drops conc. H2SO4 were added it showed blue colour.

Tests for Saponin Glycosides:
Foam test: The drug extract or dry powder was shaken vigorously with water. Persistent foam was observed.
Haemolytic test: Test solution was added to one drop of blood placed on a glass slide. It was observed whether haemolytic zone appeared.

Tests for coumarin glycosides: Test solution when made alkaline, showed for blue or green fluorescence.

Grignard's test: Strips of sodium picrate filter paper were inserted between split cork stoppers which were fitted into the neck of the test tube containing a small amount of powdered drug in the water. Care was exercised to see that the paper did not touch the inner side of the test tube. The content was warmed for half an hour. The red colour of the strips indicated the presence of cyanogenetic glycosides.



Tests for Flavanoids:

Shinoda test:
To dried powder or T.S., 5 ml 95% ethanol, few drops concentrated HCl and 0.5 g Mg turnings were added. Pink colour was observed. To a small quantity of residue, a lead acetate solution was added. It gave rise to yellow coloured precipitate. Addition of the increasing amount of NaOH to the residue exhibited yellow colouration, which was decolourised after addition of acid.

Ferric chloride test:
To T.S., few drops of ferric chloride solution were added. Intense green colour was observed.

Tests for Alkaloids:

Dragendroff's test: To 2–3 ml T.S. few drops Dragendroff's reagent were added an orange brown precipitate was observed.

Mayer's test: 2 – 3 ml T.S. mixed with a few drops Mayer's reagent gave rise to precipitate.
Hager's test: 2–3 ml T.S. with Hager's reagent showed yellow precipitate.

Wagner's test: 2 – 3 ml T.S. with a few drops of Wagner's reagent gave reddish brown precipitate observed.

Test for gums and mucilages:
The test solution was hydrolyze using dilute HCl and Fehling's or Bendict's test was performed as mentioned above. The red colour confirms the presence of gums and mucilage.

Test for Triterpenoids:

Salkowski test: Few drops of concentrated H2SO4 were added to the test solution, shaken and on standing the lower layer turned golden yellow.

Liebermann–Burchard test: To the test solution of the extract, few drops of acetic anhydride were added and mixed well. Then 1 ml of concentrated H2SO4 added from the sides of the test tube, a red colour was produced in the lower layer indicating presence of triterpenes.

Solvent partitioning of the crude extract :
The concentrated crude extract was sequentially extracted with equi-propational of water : n-hexane (3×400 ml), dichloromethane (3×800 ml) and finally with ethyl acetate (3×400 ml). The solvent fractions were separately concentrated to dryness in vacuo by using rotary flash evaporator to give four solvent fractions like water, n-

hexane (Hex), dichloromethane (DCM), and ethyl acetate (EtOAC).

Determination of in vitro cytotoxicity (MTT assay):

MTT assay: Medium HeLa (human cervical carcinoma) cell line obtained from Pune, was used in this study. Cells were cultured in liquid medium (RPMI1640) supplemented 10% Fetal Bovine Serum (FBS), 100ug/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 and 95% air at 37ºC. Cell proliferation was ensured by performing MTT assay. It is a colorimetric method used to determine cytotoxicity of medicinal agents as well as toxic materials. In mitochondria of live cells, Yellow colored MTT (3-(4, 5-dimethylthiazole 2-yl)-2, 5 diphenyl tetrazolium bromide, tetrazole) is reduced to purple formazan only when the mitochondrial dehydrogenase enzymes are active. Usually dimethyl sulphoxide is used as a solubilization solution to dissolve the insoluble formazan into a purple colored solution (Mosmann et al, 2018).

Procedure

1 x 105 cells /ml of Hela Cells were seeded in a 96 well plate and was incubated for 48 hours in a CO_2 incubator at 37oC. Concentrations of phlorotannins rich extract vise 5µM, 10µM, 20µM, 40µM, 50µM, and 100µM were added to all cells and incubated for an additional 24 hours in a CO2 incubator. And then sample and standard (IC50 concentrations) were added to each well and incubated for an additional 24 hours in a CO2incubator at 37oC. To each well 50 µl of MTT reagent (5 mg / ml) were added and the plates were again incubated for 24 hours at 37oC. 100 µl of DMSO solution were added to all wells when the purple precipitate was clearly visible when viewed under a microscope. By to and fro movement the formazan crystals formed were dissolved completely and the absorbance of all the wells including the blank was measured at 540 nm. The correction value was calculated using the formula Corrected value = OD at 540 nm - OD at 690 nm using Spectrophotometer. Cell and sample control was also included in each assay to compare the fall of cell viability in anti-inflammatory assessment. (Claria J et al, 1995)

Cell viability = OD of test / OD of standard x 100

In-vitro anti-arthritic activity[45]

Arthritis, an autoimmune disease is characterized by swelling, pain and inflexibility. It may progress in to multisystem inflammation with joint destruction. It is an inflammation of synovial joint due to immune mediated response. Denaturation of proteins, membrane lysis are the causes of production of auto-antigens in arthritic conditions. As part of the investigation the ability of the phlorotannin rich extract of Sargassum wightii to inhibit protein denaturation were studied in the present study.

Inhibition of Protein denaturation method

The test solution was prepared by taking 0.45 ml of Bovine Serum Albumin (BSA – 5 % w/v aqueous solution) and 0.05 ml of phlorotannin rich extract of Sargassum wightii in various concentrations.

- The control solution was prepared by adding 0.45 ml of BSA and 0.05ml of distilled water.
- Product control consists of 0.45 ml of distilled water and 0.05 ml of phlorotannin rich extract of Sargassum wightii in various concentrations.
- Standard solution consists of 0.45 ml of BSA and 0.05 ml of diclofenac sodium solution in various concentrations.
- The pH of the above solutions were adjusted to 6.3 using 1NHCl and incubated at 37^0C for 20 minutes.
- Then the resultant solutions were heated at 570C for 3 minutes.
- The solutions were then cooled, 2.5 ml of phosphate buffer was added and the absorbance of resultant mixtures were read at 660 nm.
- The obtained test results were compared with the control solution and standard Diclofenac sodium.
-

The percentage inhibition of protein denaturation was calculated as follows:

% Inhibition = 100- (OD of test solution – OD of product control) x 100

OD of test control

Statistical analysis:

Results were expressed as mean± standard error mean multiple comparisons of the significant analysis of variance (ANOVA) followed by the Dennett's test as post parametric test using computer based fitting program (Prism graph pad 5.0). A p value of <0.05 was considered as statistically significant[46].

3. Results and Discussion

Table 1: Ash Value of marine brown algae *Sargassum wightii*

Ash values % w/w*			
Part of plant used	***Total ash***	***Water soluble ash***	***Sulphated ash***
Brown algae	4.12%	1.91 % w/w	0. 03% w/w

Table 2: Extractive Value of marine brown algae *Sargassum wightii*

Part of plant used	**Ethanolic soluble**	**Water soluble**
Brown algae	26.72% w/w	22.12%w/w

Table 3: Phytochemical evaluation of ethanolic extract of marine brown algae Sargassum wightii

S.No.	phytochemical constituents	Inference
1.	Alkaloids	-
2.	Polysaccharides	+
3.	Glycosides	-
4.	Saponins	-
5..	Phytosterols	-
6.	Phenols	+
7.	Tannins	+
8.	Flavanoids	+
9.	Proteins	+
10.	Amino acids	+
11.	Diterpenes	-

Table 4: Extraction and fractionation of phlorotannins from *S. wightii*

Samples	yields
Ethanolic extract	20.452 g
Water	2.56g
n-hexane	10.14
Dichloromethane	4.52 g
Ethyl acetate	1.202 g

Table 5: Total phenolic contents in the ethanolic extract of marine brown algae Sargassum wightii expressed in terms of mg of phloroglucinol equivalent (mg of phloroglucinol /g of extract).

Sample	Ethanolic extract
Total polyphenols content (mg phloroglucinol /g extract)	4.15 ± 0.13

Table 6: In vitro antioxidant activity of phlorotannins rich extract of *Sargassum wightii* by DPPH method

Concentration (µg/ml)	% inhibition	IC_{50}/(µg·mL−1)
Control	-	-
Phloroglucinol	89.18± 0.05	4.3± 0.13
Phlorotannin rich extract	87.48± 0.15	19.15± 0.85

Table 7: In vitro antioxidant activity of phlorotannins rich extract of *Sargassum wightii* by Hydrogen Peroxide Method

Concentration (µg/ml)	% inhibition	IC_{50}/(µg·mL−1)
Control	-	-
Phloroglucinol	56.42± 0.23	29.16 ± 1.08
Phlorotannin rich extract	41.18± 0.46	59.56 ± 2.56

Table 8: In-vitro antioxidant activity of phlorotannins rich extract of Sargassum wightii by Hydroxyl radical

Concentration (µg/ml)	% inhibition	IC_{50}/(µg·mL−1)
Control	-	-
Phloroglucinol	48.86± 0.15	59.4 ± 0.75
Phlorotannin rich extract	41.62± 0.92	41.6 ± 0.38

Table 9: In vitro antioxidant activity of phlorotannins rich extract of *Sargassum wightii* by Nitric oxide

Concentration (µg/ml)	% inhibition	IC_{50}/(µg·mL−1)
Control	-	-
Phloroglucinol	56.18± 0.025	38.0 ± 0.08
Phlorotannin rich extract	48.25± 0.45	35.2 ± 0.09

Table: 10 In vitro Anti-arthritic activity of phlorotannins rich extract of Sargassum wightii by inhibition of Protein Denaturation method

Concentration	% Inhibition of Protein denaturation		
(µg/ml)	Phlorotannin rich extract	Crude extract	Diclofenac sodium

50	59.6 ± 0.82	23.52 ± 0.76	77.40 ± 0.84
100	65.29 ± 0.68	35.09 ± 0.65	83.77 ± 0.64
200	76.5 ± 0.80	42.46 ± 0.63	88.68 ± 0.56
500	82.67 ± 0.38	50.92 ± 0.70	91.83 ± 0.28

Figure 3: TLC analysis of ethanolic extract of marine brown algae Sargassum wightii. chloroform: methanol: water (9:1:1).

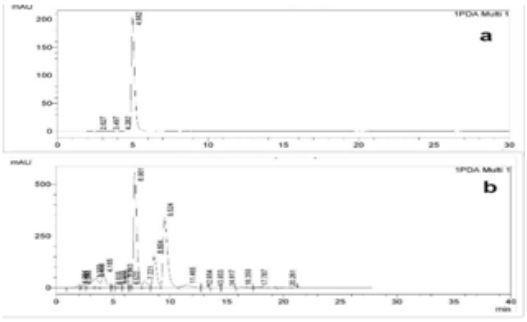


Figure 4: High-performance liquid chromatography profile of a) phloroglucinol and b) ethanolic extract of marine brown alga *Sargassum wightii* (254 nm). Mobile phase :methanol: water (95:5), 1ml/min flow rate.

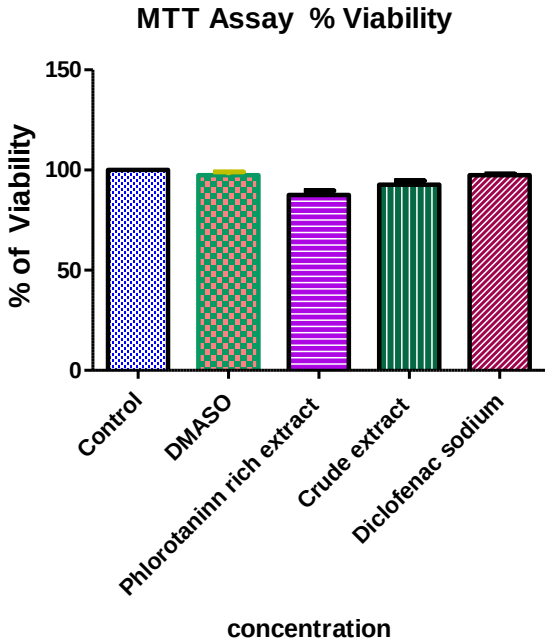


Figure 5: MTT Assay

Discussion

Extraction and fractionation of phlorotannins from *S. wightii* : A yield of about 20.452 g was obtained from the ethanolic extract of *S. wightii*. The ethanolic extract was further fractionated with water, n-hexane, dichloromethane, and ethyl acetate, which yielded 2.56g, 10.140 g of n-hexane fraction, 4.52 g of DCM fraction and 1.202 g of ethyl acetate fraction. The ethyl acetate fraction was further fractionated with a silica gel (60-120) by column chromatography and 8 fractions were collected. All the fractions were subjected to TLC, leading to the production of 4 subfractions wise E_1, E_2, E_3 and E_4.E_4 fraction produced maximal absorption in uv at 245-280nm.

Total phenolic contents:

The phenolics, which include polyphenols and phlorotannins, were estimated in the present study. In the present study, the total phenolic content of ethanolic crude extract and ethyl acetate fraction was determined by using the Folin–Ciocalteu method and the results are presented. The total phenolic content of the crude extract and ethyl acetate fractions was expressed as mg phloroglucinol equivalent per gram of the extract or fraction. The ethyl acetate fraction has the highest amount of phenolic compounds 15.54 mg and crude extract has 4.34 nmg phloroglucinol equivalent per gram .

HPLC analysis of isolated fractions :

E_4fraction was analyzed by using HPLC system consists SHIMADZU C18 column stationary phase using mobile phase methanol: water ratios of 85:15 flow rate 1ml/min wavelength at 254 nm [51]. For the E_4 fraction a peak obtained at retention time 2.704 min and for standard phloroglucinol at 2.762 min. tailing factor of the sample is 1.430 whereas phloroglucinol 1.078 .

***In vitro* Anti-arthritic activity of phlorotannins rich extract of *Sargassum wightii* by inhibition of Protein Denaturation method.**

The brown algal phlorotannin rich extract of *Sargassum wightii* showed potent *in vitro* anti-arthritic activity by inhibition of protein denaturation method. The results are tabulated in the Table 10. Phlorotannin rich extract of *Sargassum wightii* showed maximum anti-arthritic activity of 82.67 % at 500µg/ml while crude algae extract showed 50.92 % compared to that of the standard Diclofenac sodium which produced a percentage inhibition of protein denaturation of 91.83.

4. Conclusion

Seaweeds have been used since ancient times as food, fodder, fertilizer and as source of medicine. The seaweeds from Gulf of Mannar have been less explored for anti-arthritic activity. The brown algae Sargassum wightii selected for the present study have been reported to have bioactive principles. They have been reported to have antitumor, anti-inflammatory, immune modulatory activities etc. The aim of the present study was to identify

bioactive principle from marine source and to determine its anti-arthritic potential. The Seaweeds Sargassum wightii (brown algae) was collected from Mandapam, Gulf of Mannar and authenticated. Determination of ash value, extractive value and crude fiber content indicated the genuineness of the seaweed. The ash value was found in the brown algae Sargassum wightii indicating the moderate amount of total minerals and good fiber content. Preliminary phytochemical analysis of both the powder and various extracts of marine brown algae Sargasum wightii was carried out which showed the presence of alkaloids, carbohydrates, saponins, glycosides, proteins and amino acids, steroids, phenolic compounds, flavonoids, terpenoids, tannins which added to its potentiality as a bioactive principle.

The phlorotannins were quantified by Folin-Ciecult method indicate the brown algae is equipped fully with poly phenolic tannins, which were further isolated by column chromatography followed by HPLC. The phlorotannin rich extract was investigated for its potency as antioxidant against various free radicals mechanism resulted as good antioxidant activity. The phlorotannin rich extract was investigated for cell viability test and in vitro anti-arthritic activity. The results of the study reveals that it potentially inhibits the protein denaturation which is important or possible mechanism in the pathophysiology of arthritis.

Finally, the present study is confirmed by noticing the ability of phlorotannin rich extract to quench various free radicals along with the maintaining the cell viability assay. And the phlorotannin rich extract significantly prevented the denaturation of the protein indicate as anti-arthritic activity. Further studies should be carried out for elucidating the mechanism of action through which the phlorotannins acting as anti-arthritic in animal models.

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