International Journal of Current Trends in Pharmaceutical Research
CODEN (USA): IJCTGM | ISSN: 2321-3760
Journal Home Page: www.pharmaresearchlibrary.com/ijctpr

Research Article

Separation and Characterization of Phlorotannins from Marine Brown Algae *Sargassum Wightii* for Antioxidant and Cytotoxic Activity

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Abstract

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. Seaweeds 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 85:15flow 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 cytotoxicitypotential 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, cytotoxicity.

Article Info

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Article History: Received 14 July 2023, **Accepted** 20 Aug 2023, **Available Online** 07 October 2023

Citation: *Prem Sundar Pokuru et al., Separation and Characterization of Phlorotannins from Marine Brown Algae Sargassum Wightii for Antioxidant and Cytotoxic Activity. Int. J. Curnt. Tren. Pharm, Res.*, 2023, 11(1): 54-59.

CONTENTS

1. Introduction

The major background of the present study is when the cells are exposed to the chemical stimulus are damaged by the generation of the free radicals followed by cell death by its toxicity. The present study was designed to verify the hypothesis that chemical induce cytotoxicity by the liberation of free radicals which further lead to the cell death or necrosis, and also *in vitro* anti-cancer or cytotoxicity activity. The current study is investigated the defensive or protective effect of isolated phlorotannins from *Surgassum wighitii* against chemical or free radical toxicity induced cytotoxicity along with potentials of anti-oxidant activity.

Botanical name: *Sargassum wightii*
Family*:* Sargassaceae
Classification:
Empire: Eukaryota
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, immunemodulatory, anticoagulant, hepatoprotective, antiviral activity etc (Subhash yende et al., 2019).

2. Methodology

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, FT-IR spectrophotometer.

Extraction of *S. wightii*

The powdered seaweed (500g) was extracted with 2L of 95% ethanol at room temperature for 72 h and filtered using Whatmann No. 1 filter paper. The crude extract was concentrated in vacuo at 40 °C by using rotary flash evaporator to about 1/4 of the original volume. The combined ethanolic extracts were evaporated under reduced pressure to a dark green semisolid i.e. ethanolic extract of *Sargassum wightii (ESW)* and it is lyophilized to further use.

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 total polyphenols content[26]

Total polyphenols content in the ethanol crude extract and ethyl acetate fraction was determined according to a modified version of the Folin-Ciocalteu method using phloroglucinol as the standard. The dried residues obtained from the extraction were diluted according to the measurable range of the spectrophotometer (*e.g.*, a 0.025−0.1 ml aliquot of extracts of soluble phenolic was mixed with 0.475−0.4 ml water). A 0.1 ml aliquot of the diluted sample was mixed in a test tube with 0.5 ml of 50% Folin-Ciocalteu reagent and 0.5 ml of water. The mixture was allowed to stand for 5 min following the addition of 2.0 ml of 20% Na_2CO_3. Samples were incubated in the dark at room temperature for 1hr and 45 min and centrifuged at 1600 × g for 10 min. Absorbance of the supernatant was measured at 730 nm using spectrophotometer. Total polyphenols contents were calculated using the standard graph plotted.

Purification of phlorotannins of *S. wightii* from the ethyl acetate fraction by column chromatography

The ethyl acetate fraction was further purified by column chromatography. The column was prepared by mixing 100 g of silica (60–200 lm mesh size) and chloroform: water (9:1), and the mixture was stirred until it became less viscous. The reaction was exothermic in nature, with the initially thick mixture loosening up after stirring, producing slurry.

The amount of silica was determined as at least 10% of the extract weight i.e. as the extract was approximately 0.1 g, 100 g of silica was used but then extra silica was added to increase the length of the column (5.0 cm diameter × 61 cm length). The mixture was then carefully poured into the column, which was agitated using rubber tubing to help expel air bubbles within the column and to facilitate the elution of compounds more evenly. The extract previously dissolved in the reaction mixture was then placed on top of the prepared column and leveled off. More chloroform: water was used to help hydrate the dried silica and to

produce an even, thin band of the extract. Then the fractions were eluted by starting with chloroform/ methanol in the following ratios successively i.e. 90:10 and 95:5. Each fraction was then collected in pre-labeled test tubes. A total of 4 fractions were collected and they were subjected to TLC.

Thin layer chromatography

Thin layer chromatography was performed on a silica gel plate (5×20 cm, Kieselgel 60F, 0.25 mm, Merck). An aliquot of each fraction was spotted on the silica gel plate with a solvent system of chloroform/ethanol/acetic acid/water (98:10:2:2 v/v). The spots were visualized by placing the silica gel plates in Iodine chamber.

Brown-Blue colored spots produced indicated that the fractions were phenolic compounds.

Determination of phlorotannins by using HPLC method

Phlorotannins were determined by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Simazdu (Waters Associates, Milford, MA, USA), a MCPD-3600 UV detector (Otsuka Electronics, Osaka, Japan) and C_{18} column (GL Science, Tokyo, Japan). Elution was performed at a flow rate of 1ml/min using ratio of methanol: water 75:25, 85:15 and 95:5 for 15 min. The universal UV detector was set at 254 nm. Phloroglucinol was used as an internal standard.

FT-IR analysis

FT-IR analysis was performed with a mixture containing powdered potassium bromide (KBr) and lyophilized ethanolic seaweed extract. The molecular functional vibrations of chemical groups present in the sample was recorded with ANALYTICAL FT-IR spectrophotometer operated at a resolution of 4 cm^{-1} ranging from 4000 to 400 cm^{-1}.

Antioxidant activity[27,28]

Antioxidant activity should not be concluded based on a single antioxidant test model. And in practice several in vitro test procedures are carried out for evaluating antioxidant activities with the samples of interest. Another aspect is that antioxidant test models vary in different respects. Therefore, it is difficult to compare fully one method to other one.

MTT assay[31]

Cell Line and Culture

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), 100 u/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO2 and 95% air at 37°C. 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*, 1983).

3. Results and Discussion

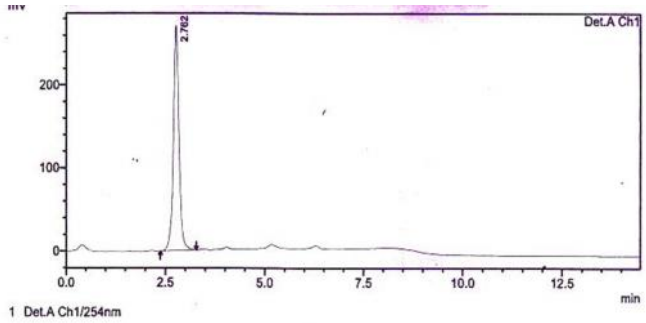

Figure 1: High-performance liquid chromatography profile of phloroglucinol (254 nm). Mobile phase methanol: water (85:15), 1ml/min flow rate.

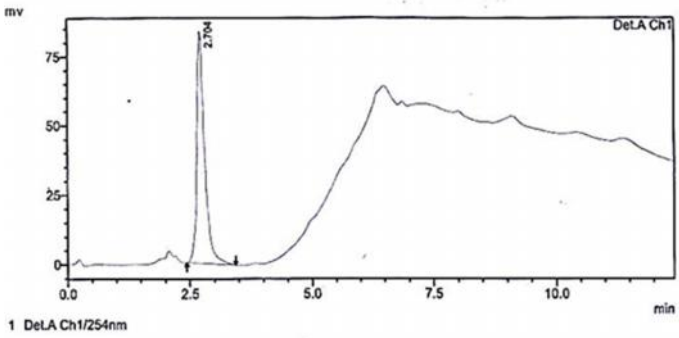

Figure 2: Separation of phlorotannins from ethyl acetate fraction (E4) of *Sargassum wightiiby* HPLC (detection at 254 nm). Mobile phase methanol: water (85:15), 1ml/min flow rate.

Table 1. *In vitro* antioxidant activity of phlorotannin 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 2. *In vitro* antioxidant activity of phlorotannin 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 3. *In vitro* antioxidant activity of *Abutilon indicum* L. 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 4. *In vitro* antioxidant activity of phlorotannin 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

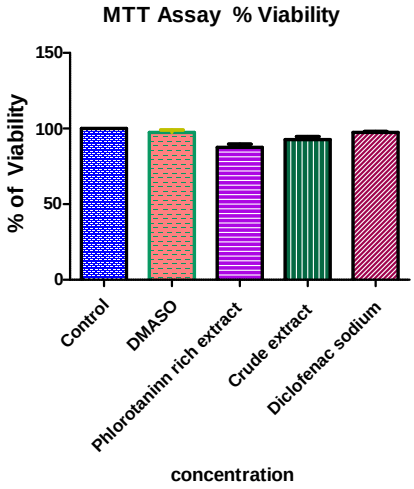

Figure 3: MTT assay

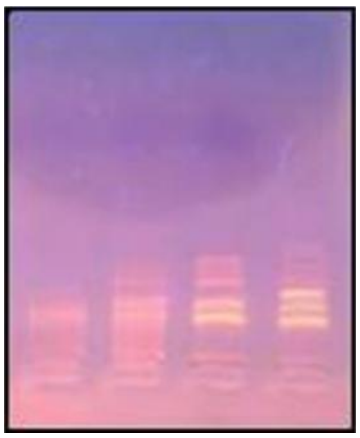

Figure 4: DNA Fragmentation analysis

Lane 1: DNA of HeLa cell line control
Lane 2: DNA of HeLa cell line + 25µg/ml of crude extract of *Sargassum wightii*
Lane 3: DNA of HeLa cell line + 50µg/ml of crude extract of *Sargassum wightii*
Lane 4: DNA of HeLa cell line + 20µg/ml phlorotannin rich extract of *Sargassum wightii*

Discussion

The Phytochemical screening of whole plant of *S. wightii*revealed the presence of phytoconstituents such as saponins, flavanoids, alkaloids, tannins and amino acids. Many researchers declared that marine brown algae having rich of phlorotannins may possess protective effect against the oxidative changes induced by chemical agents or by any free radicals (Purnima Ashok et al., 2018). Due to the presence of the above mentioned phytoconstituents, ethanolic extract of *S. wightii*are exhibiting free radical scavenging activity. MTT reduction assay is to assess the range of non-cytotoxic concentrations for which the exposure to the purified extracts, the present study found that no extract either phlorotannin rich or crude extract did not significantly affect cell viability. The evaluation of the phlorotannin rich extra as well crude extract-treated cells viability was not significantly affected compared to the DMSO and control cells by MTT assay, indicated that, even at the highest concentration, phlorotannin purified extracts did not affect mitochondrial activity**.** The purpose of the present study was to evaluate the effectiveness of phlorotannins or polyphenols extracted from brown algae in inhibiting carcinogenesis. The present work demonstrated that treatment with phlorotannins rich

brown algae inhibited the growth of HeLa both in MTT assay and Trypan blue assay , and the damage of DNA was supported by DNA fragmentation by gel electrophoresis. Based on this study, it is strongly believed that the cross killing may have occurred due to cytotoxic activity of the samples against the cell lines. Such phlorotannins are phytochemical constituents acting as major components from some brown seaweed which are responsible for the potential cytotoxic activity.

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. 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. From the present findings, it can be concluded that the crude and phlorotannin rich extract *of Sargassum wightii* showed toxicity against cervical carcinoma HeLa cell line in all the cytotoxic assays studied. The results showed moderate toxicity toward cancerous cell line. Hence the extracts need to be thoroughly studied using animal models.

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