VOLUME 11 ISSUE 1 2025

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 11, No. 1, 681-701 (2025)

International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

In Vitro Antioxidant and Anticancer Potentials of Sulfated Polysaccharides from *Chnoospora implexa* J. Agardh: A Sustainable Approach

Viswanathan Shyamala[1,2], Babu Rajesh[3], Natarajan Anandakumar[4], Elamvazhuthi Priya[5], Nallamuthu Thangaraju[2] and Mohammed Riyaz S.U.[6,7]*

[1]Department of Microbiology, Annai Violet Arts and Science College, Menambedu, Ambattur,Chennai - 600 053, Tamil Nadu, India

[2]Centre for Advanced Studies in Botany, University of Madras, Chennai 600 025, Tamil Nadu, India

[3]Sai Biotech, Hosur 635109, Tamil Nadu, India

[4]Department of Education, The Gandhigram Rural Institute (Deemed to be University), Gandhigram, Dindigul, Tamil Nadu, India

[5]TICEL Bio Park Limited, TIDCO Centre for Life Sciences, CSIR Road, Taramani, Chennai 600 113, Tamil Nadu, India

[6]PG & Research Department of Biotechnology, Islamiah College (Autonomous), Vaniyambadi 635752, Tamil Nadu, India

[7]Department of Prosthodontics, Saveetha Dental College & Hospitals, Saveetha Institute of Medical and Technical Sciences (Saveetha University) Chennai - 600 077, Tamil Nadu, India

**Corresponding Author*

Received: 17th July, 2024; **Accepted:** 25th October, 2024; **Published online:** 31st May, 2025

https://doi.org/10.33745/ijzi.2025.v11i01.073

Abstract: The present study includes synthesis, characterization, and biological efficacy of sulfated polysaccharides namely fucoidan from the thallus of *Chnoospora implexa* J. Agardh which is extracted with 0.1 M HCl and processed by DEAE cellulose to yield portions of fucoidan with molar mass of 20, 30, and 25 kDa, serially. The yields of fucoidan in F1, F2, and F3 are 18.3%, 27.7%, and 15.1%, respectively. F2 has the greatest sulphate concentration (23.7%), followed by F3 (12.1%) and F1 (7.8%). Following this, the fractions' *in vitro* antioxidant activity was determined by employing the superoxide radical-scavenging test and hydroxyl radical-scavenging assay at different doses in g/m. The analysis demonstrated outstanding antioxidant activity for all the three fractions, with the F2 fucoidan showing the highest antioxidant activity. Further analysis of anticancer activity of F2 fucoidan was conducted on human breast cancer (MCF-7) cells using the MTT assay, LDH assay, Phase-contrast fluorescence microscopic analysis, DAPI staining assay and cell cycle analysis. Western Blot analysis, Caspase enzyme activity test and DNA fragmentation assay were used to investigate the apoptosis. From the findings of the analysis, the F2 fraction had an outstanding anticancer effect on MCF-7 cells, with an IC_{50} value of 500 µg/ml.

Keywords: *Chnoospora implexa*, fucoidan, Apoptosis, Cell cycle, DNA fragmentation, Western blot

Citation: Viswanathan Shyamala, Babu Rajesh, Natarajan Anandakumar, Elamvazhuthi Priya, Nallamuthu Thangaraju and Mohammed Riyaz S.U.: *In vitro* antioxidant and anticancer potentials of sulfated polysaccharides from *Chnoospora implexa* J. Agardh: A sustainable approach. Intern. J. Zool. Invest. 11(1): 681-701, 2025.

https://doi.org/10.33745/ijzi.2025.v11i01.073

Introduction

Seaweed is a natural blessing with biologically productive resources and a wealth of bioactive compounds. Polysaccharides generated from marine algae have received a lot of interest due to their use in the diet, beautifying products, and pharmaceutical commerce. Several marine algae organisms have undeniably identified the origins of glycoproteins and polysaccharides of immune-stimulant, antitumoral, and antiviral function (Fouad Abdel *et al*., 1974; Costa *et al*., 2010; Campo *et al*., 2015;). In holistic medicine, seaweeds have been used for alleviative purposes such as gall stones, renal trouble, scabies, psoriasis, asthma, heart disease, ulcers, and cancers (Kwon Kim *et al*., 2010; Wijesekara *et al*., 2011). The advantages of seaweeds include reducing the blood cholesterol, remedy for genitourinary, reproductive disorders, antibiotic and anti-ageing properties (Matou *et al*., 2002). Polysaccharide that is present in marine algae and marine organisms is Fucoidan. Fucoidans, which produce L-fucose as the major sugar unit, are heavily sulfated polysaccharides present in brown algae cell walls (Ponce *et al*., 2003). They have a main backbone made up of 1, 3-linked sulfated L-fucose, with a repeating series of contrasting (1→3) and (1→4) glycosidic bonds (Berteau *et al*., 2003; Luo *et al*., 2010). Studies reported on *in vitro* analysis of cancer cells by different methods showed that fucoidans effectively inhibited proliferation (Ermakova *et al*., 2011). Chemotherapy, which is now used to treat cancer, uses chemicals that kill both healthy and malignant cells. Utilizing natural sources for treating cancer is nontoxic and economically viable route. The second most prevalent cancer in people is the Breast cancer. It starts primarily from the epithelial portion of the lymph gland in the breast tissues that originate in the breast. Genetic and epigenetic mutation aggregation leads to the transformation of intact breast cells into cell cancer. The bio-activeness of Fucoidan from marine algae depends on its molecular weight, sugar form, sulfate quality, connectivity type, and molecular geometry (Koyanagi *et al*., 2003, Yang *et al*., 2008). The marine species of *Chnoospora implexa* is economically important marine brown seaweed that is vastly available in Rameswaram, India (Coordinates: 9.288°N 79.313°E). Indian coasts have rich sources of various economically important seaweeds in abundance in the Eastern Coastal region of South India. *Chnoospora implexa* J. Agardh's biochemical examination reveals the existence of a significant amount of carbohydrates, coupled with less protein, fat, and ash content, indicating that this species is suitable for human consumption (Shyamala *et al*., 2014; Rashedy *et al*., 2021). *Chnoospora implexa* J. Agardh oceanic brown seaweed usage in the food industry could increase if it is used for its potential as a food or additive. There is potential benefits and applications of seaweed-derived polysaccharides, particularly fucoidan, extracted from the marine brown seaweed *Chnoospora implexa* J. Agardh. Seaweed, also known as marine algae, is a diverse group of marine plants that thrive in aquatic environments. They have long been recognized as valuable resources due to their rich bioactive compounds and biologically productive properties. Polysaccharides extracted from marine algae have gained significant attention across various industries, including food, cosmetics, and pharmaceuticals, owing to their unique properties and potential health benefits. Studies have identified glycoproteins and polysaccharides in marine algae with immune-stimulating, antitumoral, and antiviral properties. These compounds have shown promise in boosting the immune system and inhibiting the growth of cancer cells. Seaweeds have a long history of use in traditional medicine for a wide range of ailments, including gallstones, renal disorders, skin conditions like scabies and psoriasis, respiratory conditions like asthma, cardiovascular issues, ulcers, and even cancer. Seaweeds have been associated with various health benefits, including the reduction of blood cholesterol levels, support for genitourinary and reproductive health, antibiotic properties, and potential anti-aging effects. Fucoidan is a specific type of

polysaccharide found in marine algae, particularly in the cell walls of brown algae. It consists primarily of L-fucose sugar units and is heavily sulfated. Fucoidans have garnered attention for their potential health benefits. Research has shown that fucoidans can effectively inhibit the proliferation of cancer cells *in vitro*. This property makes fucoidans an attractive candidate for cancer therapy, especially as they offer a potentially non-toxic and cost-effective alternative to chemo-therapy. Breast cancer is a prevalent form of cancer, and it has become a significant focus of research. Fucoidans, including those derived from *Chnoospora implexa*, have been studied for their potential anticancer effects on breast cancer cells, such as MCF-7 cells. The bioactivity of fucoidan depends on various factors, including its molecular weight, sugar composition, sulfate content, glycosidic bond patterns, and molecular structure. These factors influence its potential health-promoting properties. *Chnoospora implexa* J. Agardh is a brown seaweed species found abundantly in the coastal regions of Rameswaram, India. Biochemical analysis has revealed that it contains significant amounts of carbohydrates, making it suitable for human consumption. Given its nutritional profile and the presence of valuable polysaccharides like fucoidan, *Chnoospora implexa* J. Agardh has the potential for increased utilization in the food industry as a food source or additive, possibly offering additional health benefits to consumers. The importance of marine algae as a source of bioactive compounds, with a particular focuses on fucoidan from *Chnoospora implexa* J. Agardh. The text highlights its potential for antioxidant and anticancer activities, especially in the context of breast cancer research.

The present study includes synthesis, characterization, and biological efficacy of sulfated polysaccharides namely fucoidan from the thallus of *Chnoospora implexa* J. Agardh which is extracted with 0.1 M HCl and processed by DEAE cellulose to yield portions of fucoidan with molar mass of 20, 30, and 25 kDa, serially. Anticancer activity of F2 fucoidan was conducted on human breast cancer (MCF-7) cells using the MTT assay, LDH assay, Phase-contrast fluorescence microscopic analysis, DAPI staining assay and cell cycle analysis. Western Blot analysis, Caspase enzyme activity test and DNA fragmentation assay were used to investigate the apoptosis.

Materials and Methods

Chemicals and reagents

Phosphate buffer saline (PBS), ethidium bromide, Acridine orange, toluidine blue O, alcian blue, alcian yellow, and acri running buffer for tris/acetate, along with DMSO (cell-culture grade), Dulbecco' Acetic acid, Fetal Bovine Serum (FBS), Penicillin/Streptomycin, MTT (dimethyl thiazolyl tetrazolium bromide), TFA, EDTA, NBT, Riboflavin, NaOH, NAD, lactic acid, SDS-polyacrylamide, and the dye 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich.

Collection of C. implexa

Fresh *Chnoospora implexa* was collected at low tides across Mandapam's shoreline in the Gulf of Mannar, India, during the post-monsoon months of January through March. Fresh biomass was properly cleaned in saltwater to remove any sand that had stuck to its surface, followed by a rinse in filtered water to remove salt, and then left to air dry for a week in the shade at room temperature. Dry biomass was crushed to a thickness of about 1 mm, seeded, and stored at room temperature.

Histochemical studies of Chnoospora implexa J. Agardh

The histochemical studies include fixation, preservation, dehydration, infiltration, block making, sectioning and staining methods (Johansen, 1940; McCully, 1966). To understand the location of the Sulphur attached carbohydrates in the *Chnoospora implexa* cell wall, these studies were performed. Fresh plant thallus samples was taken for freehand sections and stained with specific bright field dyes like Alcian blue, Toluidine blue 'O' (Parker *et al*., 1966) and fluorochromes like Alcian yellow (McCully *et al*., 1966), Acridine orange (Conn *et al*., 1966) to localize sulfated polysaccharides.

Extraction of sulphated polysaccharides

Using a technique described by Kylin *et al*. (1913), the sulphated polysaccharides (SPs) were isolated from the thallus of the *C. implexa* alga. 100 g of *C. implexa* thallus algal dry powder was soaked in a 7:3 solvent combination for two days while being agitated at 200 rpm in a shaker (Remi, Mumbai). For the dry biomass to completely discolor and degrade, the process was carried out twice. Before the pellet was retrieved, the defatted biomass powder was dissolved in 0.1 M HCl (1 L) and incited at the ambient temperature of 23 °C for 24 h. Repeating the extraction process led to a second extraction of the pellet. The supernatants have been collected after the pellet has been separated. Using two volumes of absolute ethanol in a 1:1 (v:v) ratio, the supernatants were precipitated while being kept at 4°C throughout the night. The precipitate was separated using centrifugation, and it was then diluted with water. A membrane was used to dialyze the fluid (Hi-Media) for two days. After that, the dialysate was frozen at 4°C. Sulphated polysaccharides (SPs) from the largest sulfate-containing thallus sections are further processed.

Sulphated polysaccharide purification through anion-exchange chromatography

Crude polysaccharides were put onto a DEAE cellulose rapid flow column (4 cm 25 cm), and then they were successively eluted using 0.1 M sodium phosphate buffer, followed by 0.2, 0.7, and 1.5 M sodium chloride solutions, all at a flow rate of 60 ml/h. Fucose was employed as a reference for the calorimetric assessment of the eluent's carbohydrate content (5 ml/tube) (DuBois *et al*., 1956). F1, F2, and F3, the three carbohydrate components were isolated, dialyzed in water, and then lyophilized for further study.

Characterization of Sulphated Polysaccharide

Chemical analysis

Using a phenolic sulphuric acid system, the calorimetric technique was used to calculate the total sugar concentration (Zemani *et al*., 2005). As detailed by Dodgson and Prince (1962), the sulphate concentration was determined using acid hydrolysis, followed by an estimation of the released sulphate. The total uronic acid level was measured calorimetrically using m-phenylphenol, with glucuronic acid serving as the reference material (Filisetti-Cozzi *et al*., 1991).

Agarose gel electrophoresis

Using agarose gel electrophoresis, the SPs' purity was evaluated according to Bjornsson (1993). Using a 0.01 M tris/acetate working buffer, SPs (1 mg/ml, dry weight) were routinely processed onto 1.0% agarose gels (pH 8.3). Using a 3% acetone solution with 0.5% (v/v) Triton X-100 in it and 0.02% (w/w) blue fluid, the gel was stained for 90 min at 90 V. O. Acetic acid was kept at a 3% concentration in the gel.

Determination of Monosaccharide Composition by TLC and HPLC

The method described by Han *et al*. (1998) and Di *et al*. (2003) was followed for evaluating the monosaccharide content of fucoidan fractions using thin layer chromatography (TLC). The resultant sulfated polysaccharides' neutral sugar content was assessed using a slightly modified porting technique. The samples (5 mg each) were put into a flask with a circular bottom containing 4 ml of trifluoroacetic acid solution (% TFA). The mixes were refluxed for two hours after spending the previous night at room temperature. The solutions were diluted to an 80 per cent TFA concentration with deionized water. The solutions were refluxed for 30 min, then diluted with deionized water to 30% TFA and refluxed for 4 h. To drain the TFA, a rotating vacuum evaporator was used. Deionized water was used to initially cleanse the solids before it was re-evaporated. Before the collected hydrolysates were neutral, this procedure was carried out repeatedly, Agilent 1100 High-Performance Liquid Chromatography (HPLC) was used to analyse the dry hydrolysate particles after they had been blended in deionized water (5 ml) using a column with a diameter of C18 (ZORBAX Eclipse XDB-C18, 4.6 mm 150 mm 3.5 m).

Molecular weight analysis

Gel filtration chromatography was used to estimate the molecular mass in moles of fucoidan. The filtered SPs (10 mg) are chromatographed with a 100 mM sodium phosphate buffer (pH 7.2) eluent on a Sepharose 6B membrane (90 cm 1.0 cm). A phenol-sulfuric acidic reaction was used to create and test 2 ml fractions on the column, which had a flow rate of 0.6 ml/min. The panel was calibrated using Dextrans (500, 70, 40, and 10 kDa).

Determination of antioxidant activity of polysaccharides

Superoxide radical scavenging activity assay

The riboflavin-light-NBT method was used to examine the systems' ability to stop nitroblue tetrazolium (NBT) from degrading photochemically during the superoxide radiation scavenging test. The reaction mixture (3 ml) contains the sample solution (1 ml), phosophate buffer (pH 7.8), 13 mM methionine, 2 mM riboflavin, 100 mM EDTA, and 75 mM NBT. The blank solution was made under the identical circumstances but without the reaction ingredients. The reaction systems were maintained in the dark with the use of a box covered with aluminum foil. A rise in absorbance at 560 nm was found, which is an indication that blue formazan was developing. Gallic acid was used as a positive control (Chia *et al*., 2015).

Hydroxyl Radical-Scavenging Assay

The hydroxyl radical-scavenging ability of fucoidan fractions was evaluated using the methodology described by Piñeiro *et al*. (2007). Fractions (1 ml, 30 M), a solution of 1,10-phenanthroline (1 ml, 1.865 10-3 M), and phosphate buffer saline (2 ml, 0.2 M, pH 7.4) were all added to a screw-capped tube in that order, and well mixed. $FeSO_4.7H_2O$ solution (1 ml, 1.865 10-3 M) was then added into the mixture. The reaction was started by adding 1 ml of H_2O_2 (0.03%, v/v). An Ultrospec 1100 pro spectrophotometer was used to measure the absorbance at 536 nm after the reaction mixture had been incubated in a water bath at 37°C for 60 minutes. The results were compared to a reagent blank. As a negative control, the reaction mixture without any antioxidants was employed, and the combination devoid of H_2O_2 served as a blank. The amount of hydroxyl radicals that were scavenged (HRSA) was estimated using the following formula:

$$HRSA\ (\%) = ((A_s\text{-}A_n) / (A_b\text{-}A_n)) \times 100$$

The absorption spectra at 536 nm for the sample, the unaltered control, and the blank after the reaction were recorded as A_s, A_n, and A_b, respectively.

Cytotoxicity of F2-fucoidan in MCF-7 cells

Cell culture maintenance

The National Centre for Cell Science (NCCS), Pune, India, made it feasible to acquire the human breast cancer (MCF-7) cell line. The cell was maintained in DMEM (Dulbecco Modified Eagles Medium), which also contained 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, as well as 2 mM glutamine and Earle's BSS. The current dosage for penicillin and streptomycin is 1 ml per litre (100 IU/100 g). The cells were kept in an incubator with humidification and CO_2 at 37°C with 5% CO_2.

Cell viability test- MTT Assay

To measure cell viability, the MTT method was used (Piñeiro *et al*., 2007,; Keqi Wang *et al*., 2008). 5000 cells were seeded using DMEM medium containing 10% FBS in 96 well plates. Less than 5% CO_2 and 95% oxygen were used to incubate the cells for 24 h at 37°C. The serum-free media was introduced after the serum medium had been removed, cleaned with PBS, and maintained in the incubator for a full hour. The serum-free medium was separated, the test plates were given SFM, and the treatment plates were given medium containing 100–1000 g/ml of fucoidan. The trial procedure approach is meticulous. With DMSO, regulatory communities have been managed. A maximum concentration of 0.01% of DMSO was applied to the medium in this sample. Five 96-well segments were separated, with one getting control

culture medium and the other receiving one of two 100 L culture media with fucoidan concentrations ranging from 100 g to 1000 g/ml. The cultures were incubated as before. After 48 h and 72 h, 100 µL of a 0.5 mg/ml each well received a dose of MTT solution. After that, the crystals were slowly dispersed using two to three pipes after the cultures had been cultured for an additional 4 h while being treated with 100 L of 20% SDS by 20 per cent dimethylformamide (DMF). Each well's absorbance was measured using a microplate reader at 650 nm. The growth inhibition rate was calculated in the manner described below:

% Growth inhibition = A_{650} nm of treated cells/A_{650} nm of control cells x 100

Lactate dehydrogenase assay

The vitality of the cell was assessed by counting the quantity of lactate dehydrogenase (LDH) enzyme produced from the wounded cell. The function of lactate dehydrogenase (LDH) depends on the simultaneous decrease of NAD+ and conversion of lactate into pyruvate when LDH is present. The dehydrogenase activity of lactate was tested by following the method described by King *et al*. (1965). In both cell lysate and the conditioned medium, lactate dehydrogenase assay was measured. With the addition of 0.1% tritonX 100 and the submission to two cycles of freezing and thawing after 24 h and 48 h of incubation, it was separately brought into the culture medium with treatment. The attached cells were lysed. The 0.5 mM, 0.1N NaOH, and 0.1 M glycine buffer in the cell lysate and medium were applied to the substrate reaction buffer, followed by 0.02 ml of NAD. Dinitro phenylhydrazine (0.02%) was used as a chromogen, and ultra-spec 400 spectrophotometers were used to detect the absorbance at 460 nm. The units are represented as M of released pyruvate/min/mg protein.

Morphology of MCF-7 cells

Phase-contrast microscopic analysis

DMSO was used as a control and F2-Fucoidan (500 µg/ml) was handled 24 h and 48 h for different durations in 96-well plates (5 µg/ml). During the treatment periods, the morphology and cell development of cells were analyzed in phase contrast mode under the microscope. A model snapped photos using an Olympus Cameda (Olympus Corp., Tokyo, Japan) equipped with C-7070 optical big zoom camera U-TVO 63 TREE C.

Fluorescence microscopic analysis

9 ml of cell suspension (1 x 10^6 cells/ml) and 1µl of a dye solution (100 mg/ml Acridine Orange (AO) and 100 mg/ml Ethidium Bromide (EtBr) in purified water) were mixed on a sterile microscope coating. Cell tumors were gathered, washed in pH 7.2 phosphate-buffered saline, and stained in 1 ml with 100 mg of apiece of the AO and EB mix stains and 100 mg of EB PBS. The cells were given a 2 min incubation period, followed by two 5 min PBS washes, after which they were seen under a fluorescence microscope (Nikon, USA) at 400X magnification with a 480 nm excitation filter (Sukirtha *et al*., 2012) .

4′ 6-diamidino-2-phenylindole (DAPI) staining assay for analyzing cell death

A nuclear staining method called DAPI was utilized to detect cell and DNA apoptotic alterations. In this investigation, the nuclear dye DAPI was seen under a fluorescence microscope to quantify apoptotic cell death. According to usual procedure, PBS containing 500 µg/ml of F2-fucoidan was used to treat MCF-7 cells for 24 and 48 h before trypsinization, extraction, and a full night's fixation at 4°C in 70% ethanol. The next day, the cells were stained with 2.5 g/ml of DAPI solution, mounted on slides, and afterwards examined under a 400X magnification fluorescent microscope to identify apoptotic features. DAPI labeling was used to demonstrate the degree of condensation of chromatin and nuclear disintegration in apoptotic cells.

Cell cycle Analysis

Using this method, 5 x 105 MCF-7 cells were cultivated on six-well Nunc (USA) plates, and the cells were incubated for 24 h. At 37 ºC and 5% CO_2, the cells were nurtured in the suitable medium. The cell layer was trypsinized, rinsed in

a chilled phosphate-saline-buffer, and fixed in 70% ethanol following 24-48 h of incubation. They were treated with cell suspensions at 37°C and propidium iodide in 30 ml (RNAase) and 20 ml (0.02 mg/ml) concentrations. The samples were then examined under the cytometer FACS caliber flow (Becton Dickinson and Co., Franklin Lakes, NJ). For each study, 10,000 cells were analyzed (Spector *et al*., 1998).

DNA Fragmentation Assay

To determine the cause of cell death, a DNA fragmentation assay was employed. For DNA extraction and agarose gel electrophoresis method was used (Chia *et al*., 2015). The grown MCF-7 cells were extracted using this method and Tri Reagent. The samples were put through a 2 h, 80 V electrophoresis process on a 0.6% agarose gel.

Western Blot Analysis

As previously mentioned, Western blot analysis has been done and cell protein lysates have been generated (Haruki T*et al*., 1999, Ramakrishnan *et al*., 2009). Using SDS-polyacrylamide gel electrophoresis, the proteins were separated. The proteins were then transformed into polyvinylidene difluoride membranes. Using improved chemiluminescence, protein bands were visible.

Caspase enzymatic activity assay

Caspases 3 and 9 protease activity in MCF7 cells was measured using a colorimetric test kit. The F2-fucoidan-treated cells were incubated at IC50 for 24 and 48 h, whereas the same amount of time was brood on the untreated cells. In order to separate the media, the cells were centrifuged at 1500 rpm for 5 min, and they underwent two PBS washes. The 50 µl ice cold buffer was added to the lysed cell pellets, which were then incubated for an hour on ice. The resultant cell lysate was spun at 10,000 rpm for a single minute. The Bradford technique was used to determine the protein content of apiece tube. To half of the 2-pastel buffer, a 50 l surfactant containing 200 g of protein in each tube was added. The substrate received 5 l of caspase before being incubated at 37 °C in the dark for 4 h. A universal microplate reader (Biotech, Inc., United States) was used to read the samples at 405 nm.

Statistical analysis

One-way ANOVA was used to statistically analyse the results. The mean difference between distinct thallus portions (Version 17) was calculated using the Duncan Multi Comparison Test and the SPSS statistical software. Data are presented as mean ± SD and $p < 0.05$ was set for statistical significance.

Results and Discussion

Extraction and Purification of Sulphated polysaccharide (*SPs*)

The present study examined the extraction of sulfated polysaccharides from the fresh thallus of C. *implexa*. The extract with a coloration of four distinct tin colors namely Alcian blue, Toluidine blue O, Alcian yellow and Acridine oranges are repented in Figure 1 (A-D). Figure 2 represents the agarose gel electrophoresis of the extract, which confirms 3 different Fucoidans, namely F1, F2 and F3.

In the thallus, the SPs showed that their distribution is similar to that of the more dispersed sulphate in the cortical areas, both homogeneous and heterogeneous (Flores *et al*., 1997, Suresh *et al*., 2013). Sulfated polysaccharides can vary in their position in the thallus between species regardless of their phylogenetic relationship. The thallus has shown optimum regeneration and sulphate material. The low molecular weight polysaccharides, fat, and pigment were removed using acetone and methanol. After SPs were precipitated by ethanol and sour water-soluble polysaccharides were isolated from the biomass of algae, dialysis was performed. Aqueous sodium chloride solutions with increasing concentrations were used to extract the resulting crude sulfated polysaccharide (F) from DEAE cellulose quick flow. The three unique fractions (F1, F2, and F3) are listed in Table 1 along with their yields and compositions. Significant sulfate contents and low uronic acid

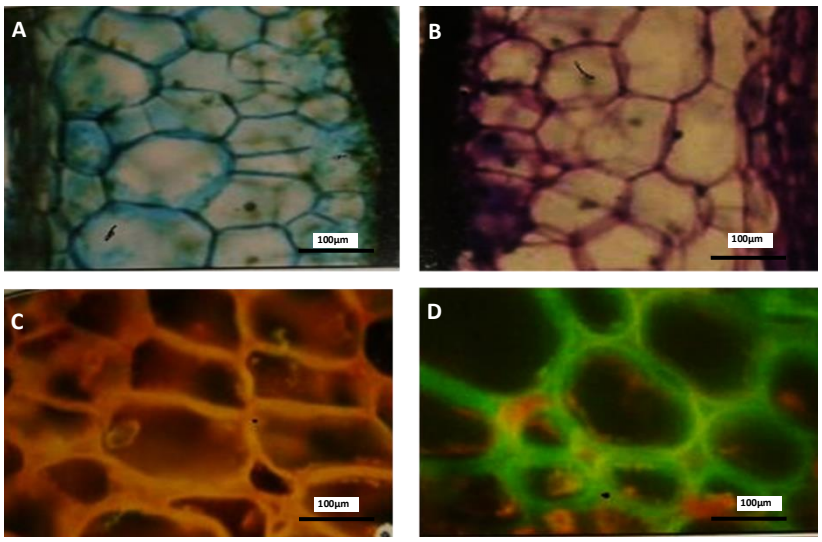

Fig. 1: Localization of sulphated polysaccharide of *Chnoospora implexa* using different stains –x360. (A) Transverse section of "Entire thallus" stained with Alcian blue; (B) Transverse section of "Entire thallus" stained with Toluidine blue O; (C) Transverse section of "Entire thallus" with Alcian yellow; (D) Transverse section of "Entire thallus" with Acridine orange.

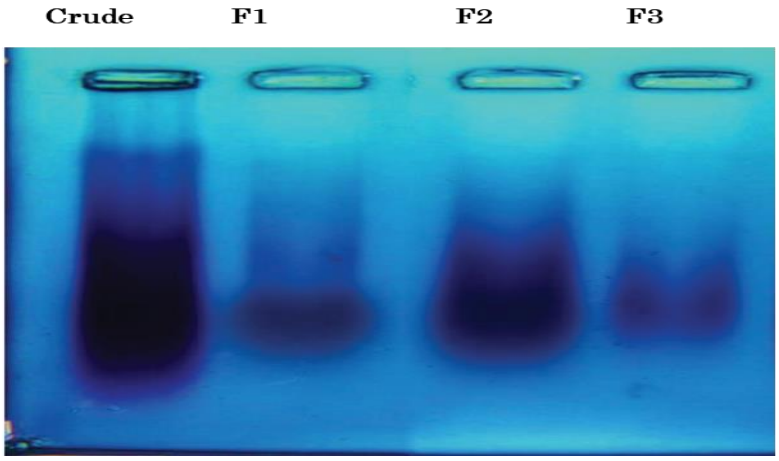

Fig. 2: Agarose gel electrophoresis analysis of Crude, F1, F2 and F3 fucoidan fractions from *C. implexa.*

concentrations were detected in all three fractions.

Molecular mass of C. implexa

The various fractions (F1-F3) of the Sepharosis 6B column's molecular weights were measured using gel permeation chromatography. The ostensible molar mass of the F1, F2, and F3 fractions were 20, 30, and 25 kDa serially, based on calibration with normal dextrans (Figs. 3, 4).

Monosaccharide identification and purity of F2-fucoidan of C. implexa

TLC and HPLC have confirmed with monosaccharides in three fractions. The distilled monosaccharide compounds (F1, F2, and F3) have been 100% TFA hydrolyzed and TLC-supplied with monosaccharide-like fucose standards. The results indicated that the F1, F2, and F3 hydrolysates in TLC were distinctly spotted, the value of which Rf almost coincides with the value

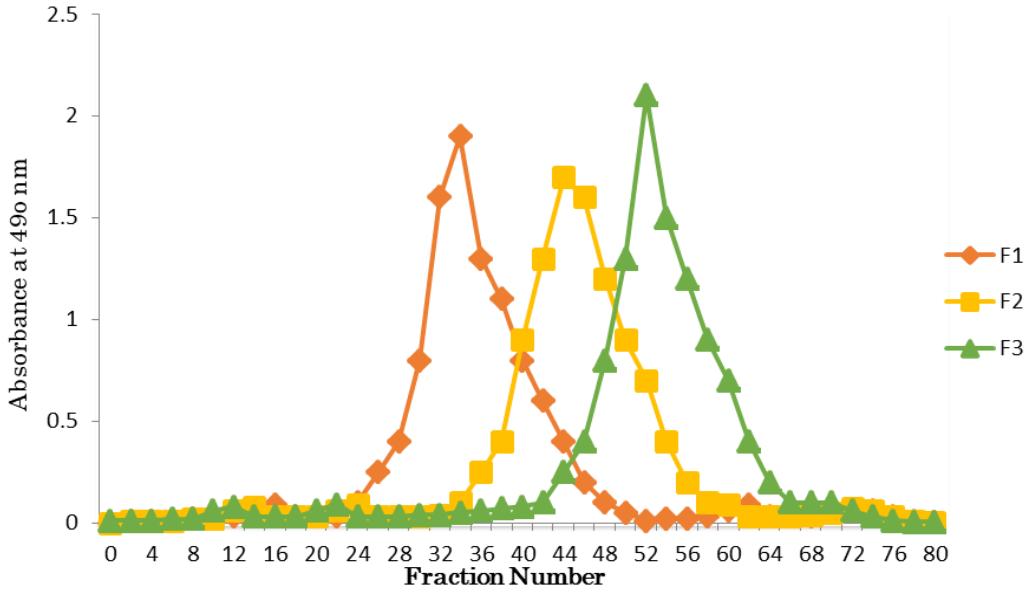

Fig. 3: Gel filtration profile of different fractions from Sepharose 6B column chromatography.

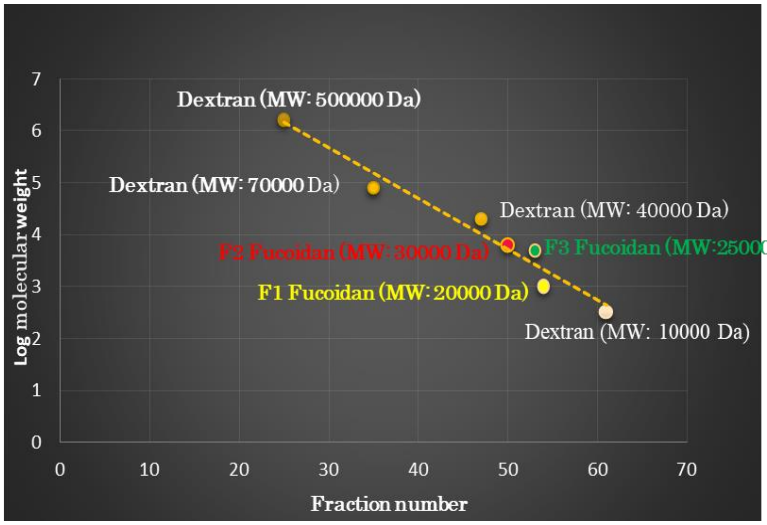

Fig. 4: Gel permeation chromatogram (GPC) of purified fucoidan calibration curve obtained from dextran standards with molecular weight determination using Sepharose 6B column.

of regular fucose Rf. F1, F2, and F3 are fucan molecules composed of polymeric fucose units (Fig. 5). Fucose, galactose, xylose, and mannose were identified as the sugars using HPLC (Fig. 6). Fucose, the primary sugar of these sucrants, was present in F1, F2, and F3 in amounts of 51.5%, 73.1%, and 65.1%, respectively (Table 1). F1, F2, and F3 contained 22.9%, 11.5%, and 13.2% of galactose, respectively.

The remaining sugars, including mannose and xylose, were eliminated from these three fractions. For the two fractions, the fraction's purity was shown in the agarose gel electrophoresis as there is only one single band showing the distilled polysaccharides recorded in the gel, which confirms the purity of the fractions (Farias *et al.*, 2008).

Antioxidant activity of fucoidan fractions of C. implexa

Free radicals are extremely reactive compounds that can be produced by radiation or metabolic processes that leave one electron unpaired. Free

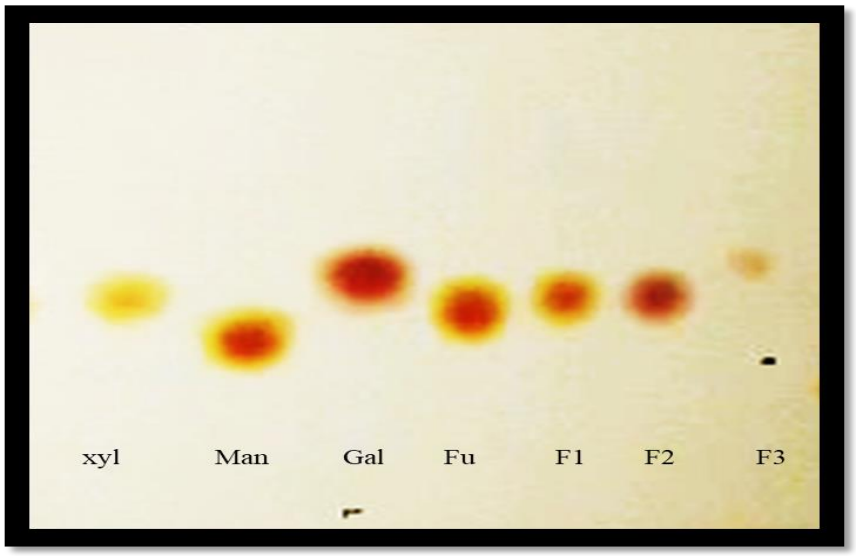

Fig. 5: Thin Layer Chromatography of different fucoidan fractions.

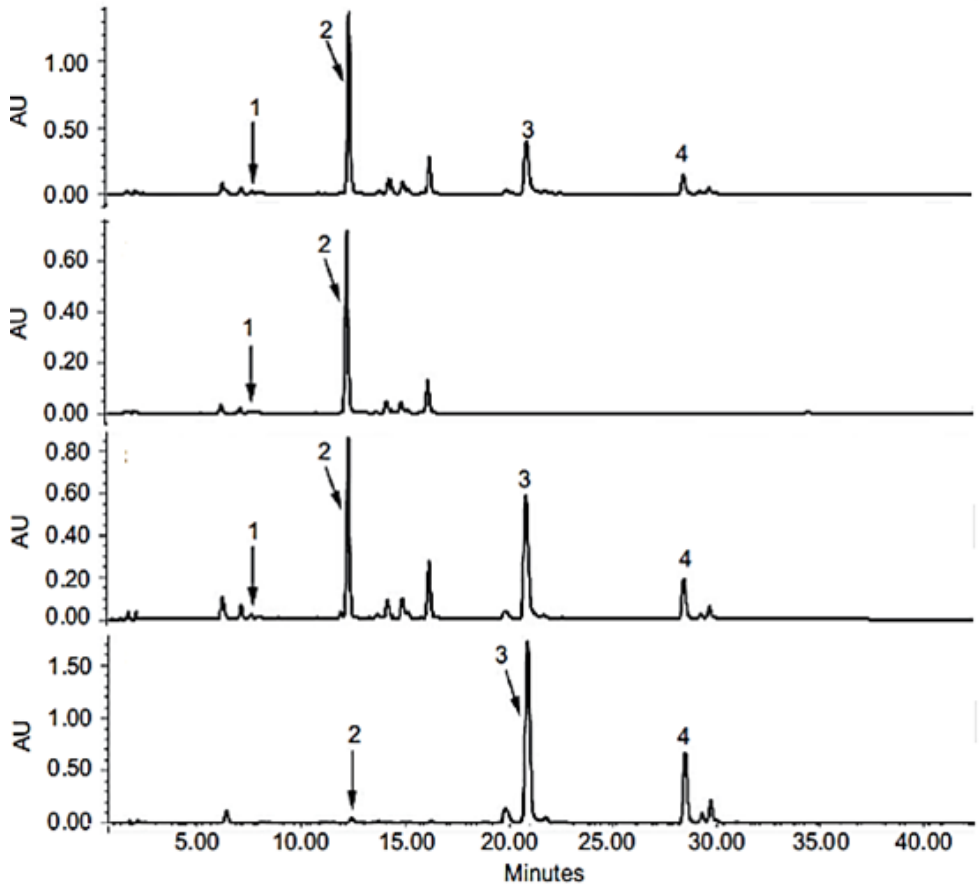

Fig. 6: HPLC Chromatograms for monosaccharide composition analysis of fucoidan fractions from *C. implexa*. (A) HPLC Chromatograms for sugar standards (1. Xylose, 2. Fucose, 3. Galactose and 4. Mannose); (B) F1 fraction; (C) F2 fraction; (D) F3 fraction.

Table 1: Yield, Chromatography composition and Molecular weight of Fucoidan fractions by Ion Exchange

Fractions	Yields (%)	Total Sugar (%)	Total Sulphate (%)	Total Uronic acid (%)	Composition of Monosaccharides (% moles)				Molecular weight (- kDa)
					Fucose	Galactose	Xylose	Mannose	
F1	18.3	53.8	7.8	20.9	51.5	22.9	4.7	6.7	20
F2	27.7	71.3	23.7	13.6	73.1	11.5	3.5	11.2	30
F3	15.1	47.9	12.1	17.3	65.1	13.2	1.9	17.9	25

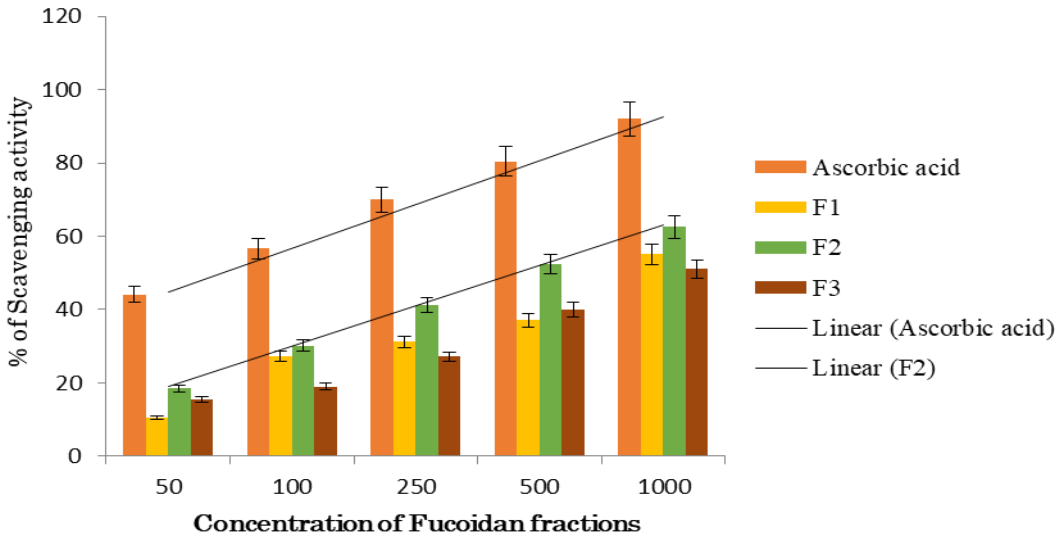

Fig. 7: Antioxidant activity- Hydroxyl scavenging activity of different fucoidan fractions (50 and 1000 g/ml) (F1-F3) of *C. implexa*.

radicals cause a chain of events that result in the lipid, protein, and nucleic acid membranes of cells as well as other components breaking down. Lipid oxidation, which harms living cells and eventually degrades the organoleptic qualities of edible foods, is mostly brought on by unstable molecules. Free radicals like peroxide, hydroperoxide, or lipid peroxyls are scavenging chemicals, and anti-oxidant substances lower levels of oxidative stress and slow/prevent issues owing to oxidative stress-related disorders (Xue *et al*., 2001; Duan *et al*., 2006).

An earlier study that used an extract from *S. plagiophyllum* rich in sulfated polysaccharides found minimal effectiveness in tests for hydroxyl and superoxide scavenging (Costa *et al*., 2010). The harmful and carcinogenic properties of many synthetic antioxidants have prompted researchers to look for natural antioxidants. Many findings described the potential effect of algae's antioxidant activity. However, there is no clear evidence on relation between the structure and antioxidant property of seaweed polysaccharides and it has not yet been clarified. Therefore, it is crucial to thoroughly research how exceptionally pure and well-defined marine polysaccharides interact with the biological system. Figures 7 and 8 display the fucoidan fractions F1, F2, and F3's hydroxyl and superoxide radical scavenging activities at different dosages between 50 and 1000 g/ml. As can be observed, both the controls (gallic acid and ascorbic acid) and the fucoidans (F1-F3) exhibit concentration-dependent radical inhibition. With higher control or fucoidan concentrations, the % scavenging activity increases. At 500 µg/ml, the controls gallic acid and ascorbic acid showed 80–90% radical scavenging effects.

When compared to the F1 and F3 fucoidan fractions at all doses examined, the F2 fucoidan fraction showed the greatest percentage of radical inhibition. The increased sulfate concentration of

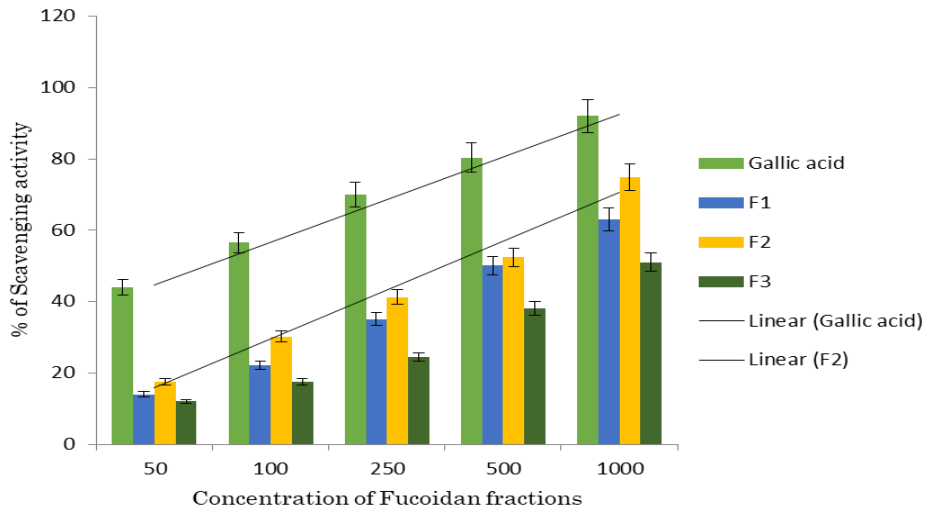

Fig. 8: Superoxide radical scavenging activity of sulphated polysaccharides. Each value is the mean ± SD of three determinations and different concentration of the same sulfated polysaccharide.

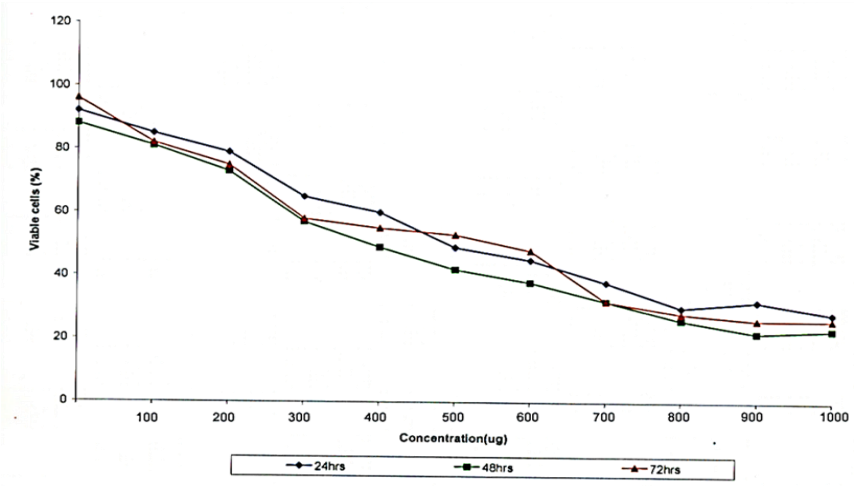

Fig. 9: Cell viability analysis of F2-fucoidan (100-1000 µM) in MCF-7 cancer cells on MTT assay at 24 h, 48 h and 72 h.

the polysaccharides may be responsible for the F2 fraction's better elimination of radicals activity.

Cytotoxicity of F2 fucoidan on MCF-7 cells

By using the MTT reduction method to determine the cell viability, the cytotoxic concentration was assessed *in vitro*. Figure 9 shows the percentages of MCF-7 cells that were still viable 24 h, 48 h, and 72 h after being treated with F2-fucoidan. It is evident that the control cell's viability (85–90%) remained constant during the whole experiment. However, it was discovered that the cell viability of F2-fucoidan treated cells had significantly decreased. In addition, the cell viability is directly proportional to concentration. The % cell viability is inversely proportional to concentrations of F2 fraction from 100 to1000 µg/ml. Only 50% of the cells were still alive after 48 hours of F2-fucoidan therapy. At 48 h, the IC_{50} values of F2- Fucoidan were 500 µg/ml. Our results demonstrate that SP has compelling cytotoxic effects on cancer cell lines as opposed to normal cell lines. Previous

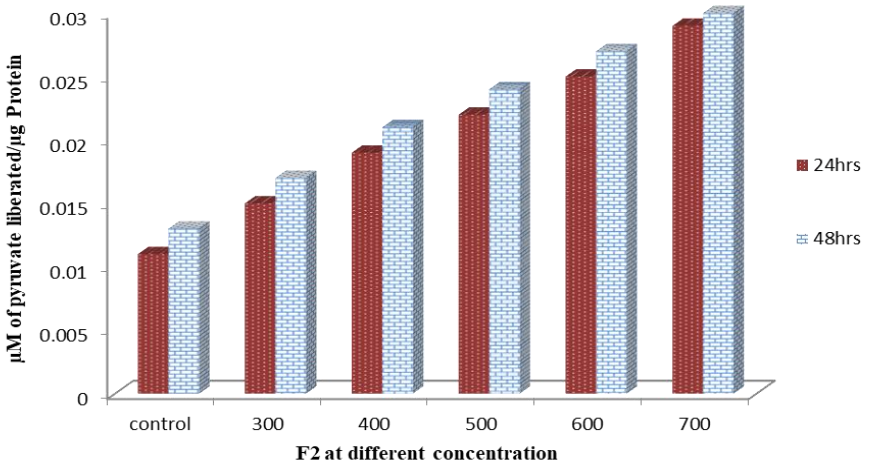

Fig. 10: Effect of F2 on cytotoxicity in MCF-7 cells assessed by LDH activity in medium for 24h and 48h, Values were obtained in triplicate, expressed as the mean ± SD and found to be statistically significant at $p < 0.05$.

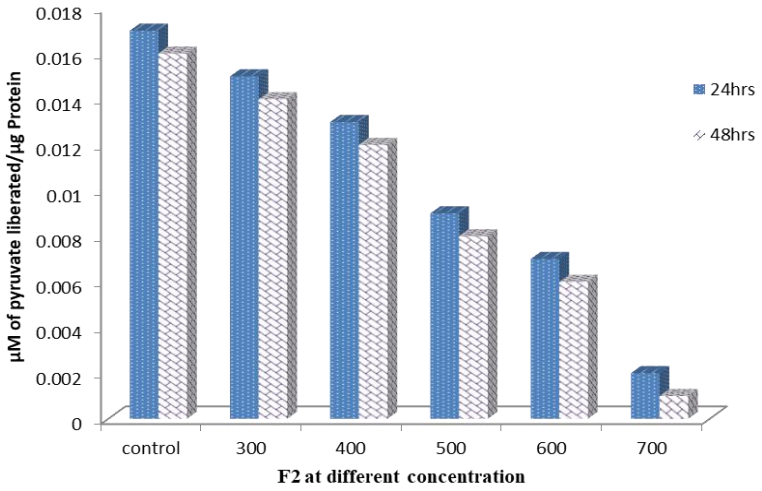

Fig. 11: Effect of F2 on cytotoxicity in MCF-7 cells assessed by LDH activity in cell lysate for 24h and 48h different concentrations (300-700 µM). Values were obtained in triplicate, expressed as the mean ± SD and found to be statistically significant at $p < 0.05$.

studies have shown that *Undaria pinnatifida* has directly suppressed the proliferation of MCF 7 cells with reduced cytotoxicity to regular cells with the cytotoxicity of two main fractions (F1 and F2) (Mak *et al*., 2014).

Lactate Dehydrogenase (*LDH*) *Assay of F2 fucoidan*

The LDH release assay is a flexible technique that may be used to assess a substance's cytotoxicity even at low doses (Sasaki *et al*., 1992; Robert *et al*., 2009). With the optimum IC50 range of 300-700 µM in both cell lysate and circumstances, the effect of LDH was assessed for the 24h and 48h treatment of F2-fucoidan (Figs. 10, 11). LDH activity in the cell lysate was substantially lower than in control cells. But in conditioned medium, LDH activity has drastically risen in comparison to control cells. The SP has also improved the

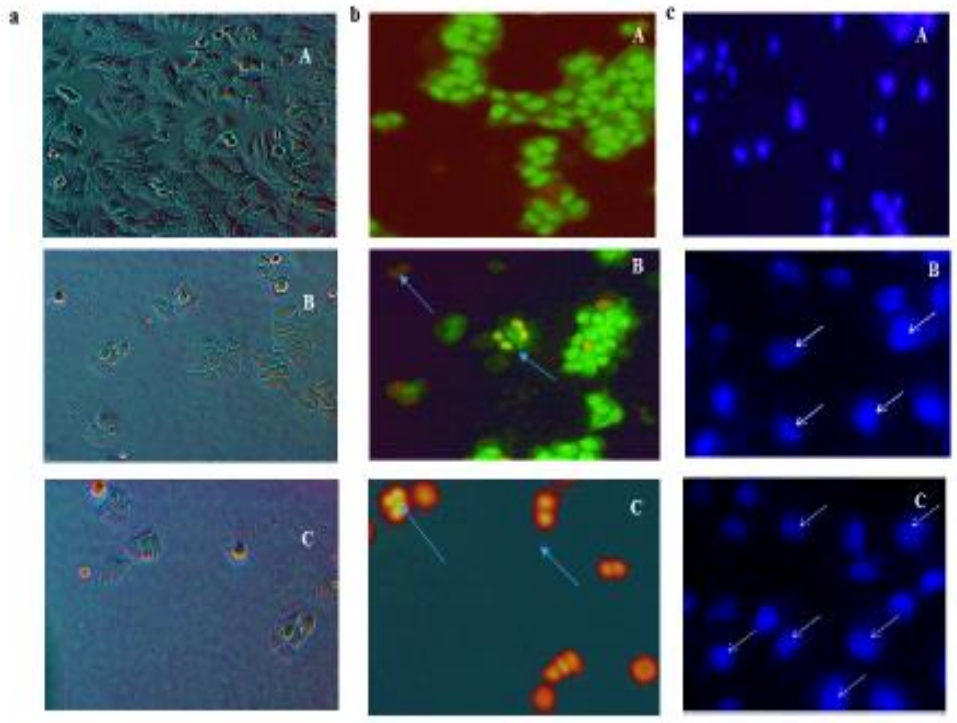

Fig. 12: Morphological characterization of MCF-7 cells under Phase contrast microscopic analysis (b): AO/EtBr staining analysis and (c): DAPI staining analysis of the induction of apoptosis in MCF-7, visualized under fluorescence microscopy. MCF-7 cells were analyzed without treatment (A) and with treatment using the IC_{50} concentrations of F2, at 24 h and 48 h (B and C, respectively).

reproducible dose-dependent release of LDH. This method along with this LDH test provides useful data on the cytotoxic effects of SPs.

Morphological analysis of MCF-7 cells with fucoidan by Phase contrast Microscopy

Untreated MCF-7 cells were observed under phase-contrast microscopy (100X) under filtered F2-Fucoidan (Fig. 12 A, B, C). After 48 h of treatment with various Fucoidan concentrations from *C. implexa*, apparent changes in cellular morphology and the separation of cells from the cell surface were noted. The degree of confluence of the concentration-based impact was found. Cell death occurred at the end of 48h with the disruption of monolayers. In comparison with untreated cells, the mitosis of cells was scarce. Because of the cytoplasmic condensation, most cells are rounded, far from their surface. The higher concentrations (500 µl) had more pronounced effects, and nearly all cells were rounded up and detached, resulting in a prominent decrease in the cell numbers.

Fluorescence microscopic observation by AO/EtBr staining

Apoptosis was found to be caused in vulnerable cells by most of the cytotoxic anticancer drugs (Dias *et al*., 2005; Qiu *et al*., 2006; You *et al*., 2010). It has been claimed that Acridine Orange's (AO) primary coloring, which may stain nuclear DNA on an undamaged cell membrane, has damaged the membrane of Ethidium Bromide (Et Br). Thus, living cells were consistently stained grey, early apoptotic cells were densely stained, showing them as green orange pieces, and late apoptotic cells were thickly stained, showing them as orange fragments. Necrotic cells had no concentrated chromatin stained with red. A uniform green fluorescence has been shown to demonstrate viable cells in untreated control for green living MCF-7 cells with natural spindle formed

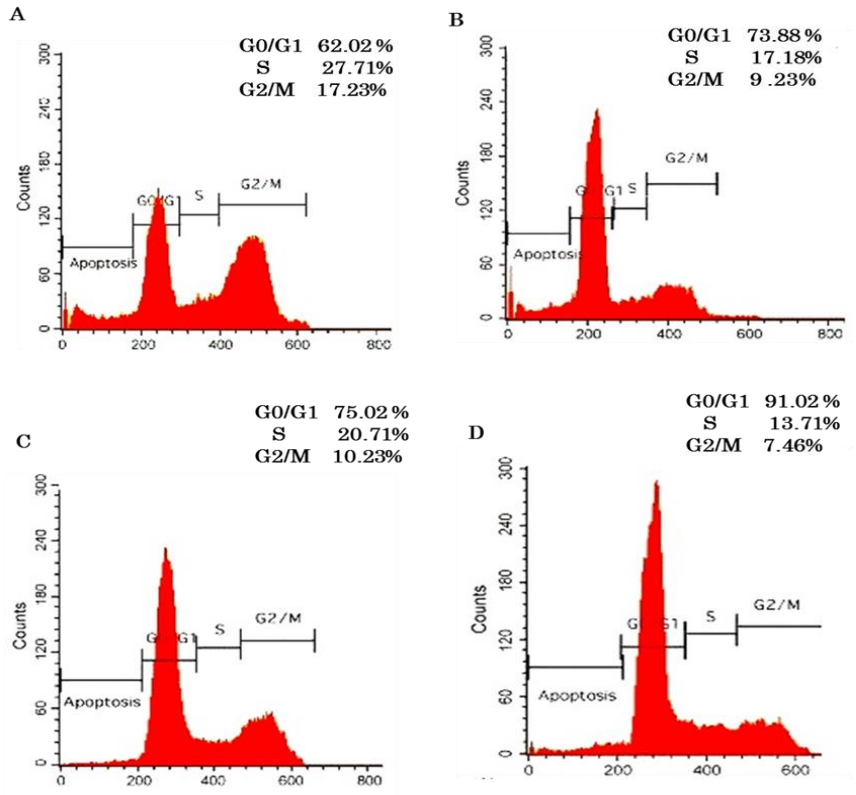

Fig. 13: Shows the distribution of the cell cycle phases after MCF-7 cells were treated with SPs of the F2 fraction. MCF-7 cells (B and D) after 24 and 48 h of exposure to their respective IC50 concentration of F2 fucoidan. (A and C) cells that were left untreated in each case. At $p < 0.05$, it was determined that the values, which were acquired in triplicate and expressed as the mean SD, were statistically significant.

morphology (Fig. 12A). In comparison, the cell nuclei showed early apoptotic (green-orange dots) and late apoptotic cells (orange dots) (Fig. 12 B), bright green cells minimized by nuclear power and chromatin condensation at 48h (Fig. 12C). Apoptotic and chromatin condensing-colored orange cells were detected. Apoptotic morphology at concentrations of 500 µg/ml is noted both early and late.

Fluorescence microscopic observation by DAPI staining

Fig. 12 (A, B, C) o revealed that compared with F2-fucoide untreated MCF-7 cells, F2-fucoidan treated MCF-7 cell morphological variations were studied through representative representations of the compact and fragmented nuclei. A nuclear morphology study of the MCF-7 cells revealed that they also exhibited typical apoptotic traits such as histone condensation, nuclear disintegration, and apoptotic bodies. The cellular deaths were recorded as time-and-dose based on all the cancer cells' cultures in *Eucheuma cottoni* and *Sargassum muticum* (Qiu *et al*., 2006).

Cell cycle analysis-Flow cytometry

F2-fucoidan effects were assessed in the cell cycles of the MCF-7 with the use of propidium iodide dyeing cytometric research. For each stage of the cell cycle (G_0/G_1, S, and G_2M), the percentage of DNA content of the control and F2-fuel-treated MCF-7 cells was observed 24 h and 48 h later (Fig. 13A, B, C, D). When MCF-7 was treated with F2-fucoidan, the proportion of cells in the Go/G1 phase increased significantly (73.88% at 24 h; 91.02% at 48 h), while the proportion of cells in the S and G2M phases declined in lockstep (17.18% at 24 h; 13.71% at 48 h; and 9.23% at 24 h; 7.46% at 48 h). The findings have shown that F2-fucoidane has several antiproliferative

functions against cancer cells.

The *in vitro* research also supports the usage of a combination of these SPs against human breast cancer cells through the F2-fucoide fraction. These differences in cancer activity may affect the molecular mass, load properties, and monosaccharide dispersion of SPs (Dias *et al*., 2005, You *et al*., 2010). Brown seaweed polysaccharides' bioactivity has previously been studied in relation to a number of structural elements, encompassing as sulphur grades, molecular weight, sulphur location, sugar shape, and glycosidic branching (Duarte *et al*., 2001). The polysaccharide fractions obtained from *C. implexa* were then tested for their sulphate content. In this analysis, the sulphate content of F2 was the highest (23.7%), led by F3 (12.1%) and F1 (7.8%). The polysaccharide F2 fractions had significantly higher anti-cancer activity against *in vitro* MCF-7 cells. Several published research indicated that the sulphated cationic protein groups in cells play an important role in suppressing cellular cancer growth (Rupérez *et al*., 2001; Li *et al*., 2008; Sundarraj *et al*., 2012). As a result, brown algal SPs that define sulfate group binding properties in cancer cells are less likely to arise. Brown algal polymer SPS is produced by a process involving intramolecular interactions, enabling sulphate regions that bind to protein surfaces on cells. The F2 fraction also included large quantities of uric acid and total sugar. Despite not having been seen, sugars and uronic acid do not cause immediate cellular damage.

It was discovered that the molar mass of three polysaccharide fractions-F1, F2, and F3 sulphated-were 20, 30, and 25 kDa serially. The F2 fraction was the most constricting of the three. Previous studies have shown that low-molecular-weight sulfated fucans are more mobile and diffusive than high-molecular-weight sulfated fucans, which enhances interactions between cancer cell components (Zemani *et al*., 2005). Strong-molecular weight fucoidane (18,6 kDa) with concentrations of 0,1 mg/ml is confirmed to have shown inhibitory activity of over 70% in CCL39 cells at another analysis conducted in *Ascophyllum nodosum*. However, another recent research showed that cell growth could be modulated in sulfated fucan polymers in various ways by their molecular weight (Xue *et al*., 2001; Li *et al*., 2008). Low molecular weight fucoidants produced from *A. nodosum* promoted cellular development in Human umblical vein endothelial cells (HUVEC) and endothelial progenitor-cells (EPC) neovascularization (Miyamoto *et al*., 2009; Chia *et al*., 2015). However, Silchenko *et al.* (2021) found that a molecular mass of 271 kDa with 29% sulfate content and a lesser molecular weight (approximately 71 kDa) reduced the growth of tumour cell colonies compared to fucoidan.

DNA fragmentation

After mitotic cell arrest was induced, either due to DNA damage or due to spindle damage, the cells of cancer struggle to suffer cytokines and continue in the cell period. The catastrophic mitotic cells are called multi-nucleated, lacking traditional apoptosis characteristics, including DNA degradation (Chan *et al*., 1999). F2-fucoidan-induced DNA fragmentation in the cell line MCF-7 is seen in Fig. 14. MCF-7 cells treated with 500 µg/ml F2-fucoidan for 24 and 48 h resulted in extensive DNA fragmentation. These findings indicated that F2-fucoidan was induced by apoptosis.

In the previous trial *Padina tetrastromatica, Tubinaria ornata* and *Caulerpa racemosa* indicated that the cause of MCF-7 cell death is due to apoptosis (Chia *et al*., 2015) and it could be the same cause in brown marine extracts observed in the present study.

Caspase activity

Caspase signage mediated acute sulphate polysaccharide, F2-fucoidan treatment and Caspase enzyme treatment were found to affect caspase signaling cascade involvement. In Caspase-3 and Caspase-9 activities, F2- fucoidan was handled at 500 µg/ml in MCF-7 cells for 24 and 48 h. After this time, cell lysate was treated under unreduced condition and treated for Western blot analysis with SDS-PAGE (12.5%)

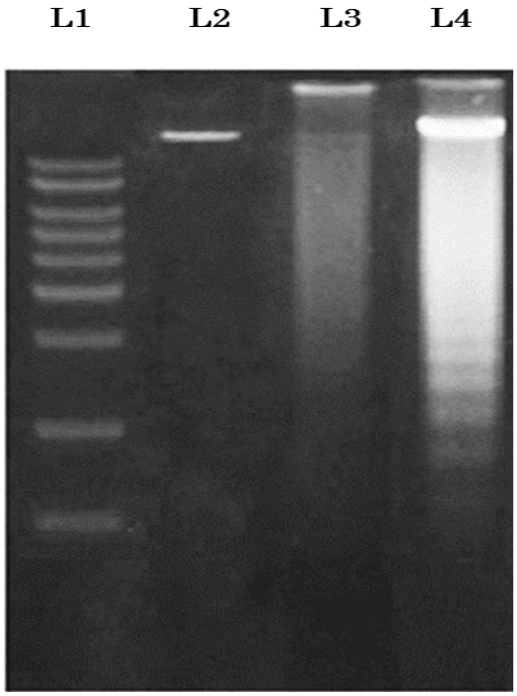

Fig. 14: DNA fragmentation analysis of MCF-7 cell line, L1- Marker; L2- Control cells; L3- Cells treated with 500 µg/ml of F2-fucoidan at 24 h; L4- Cells treated with 500 µg/ml of F2-fucoidan at 48 h.

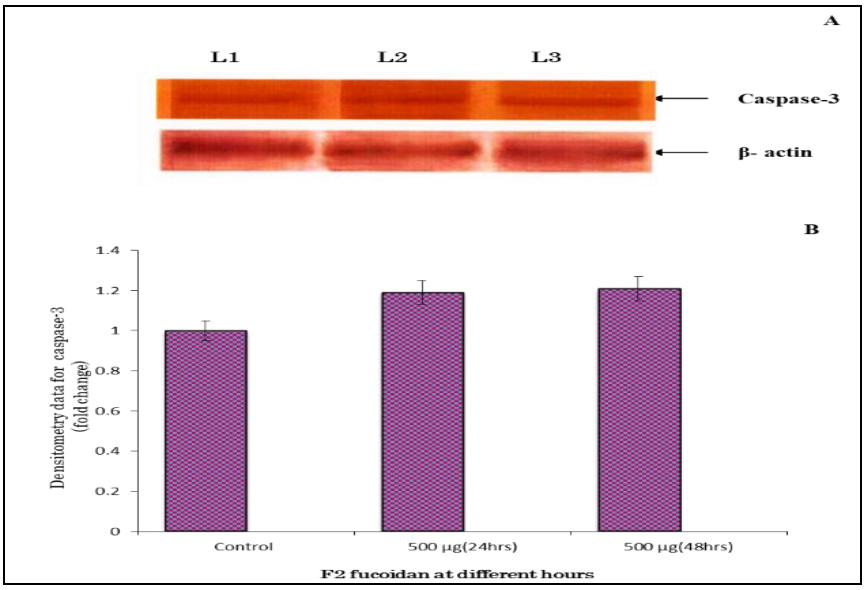

Fig. 15: (A) Representative Immunoblot expression of caspase-3 and β-actin to check equal protein of Control and F2-fucoidan treated cells.L1- Control MCF-7 cells, L2- Cells treated with 500 µg/ml of F2-fucoidan at 24h, L3- Cells treated with 500 µg/ml of F2-fucoidan at 48h. (B) Densitometry analysis of p53 western blotting. Densitometry data present as "fold change" as compared with control. Results are expressed as mean ± SD* $p<0.05$ vs control.

(Figs. 15, 16). In the analysis, apoptotic protein expression in F2-fucoidan-treated cells increased significantly in 48 h compared with control cells (Lane 1 and 3). The treated cell for 24 h nevertheless expressed a low apoptotic protein concentration (Lane 2).

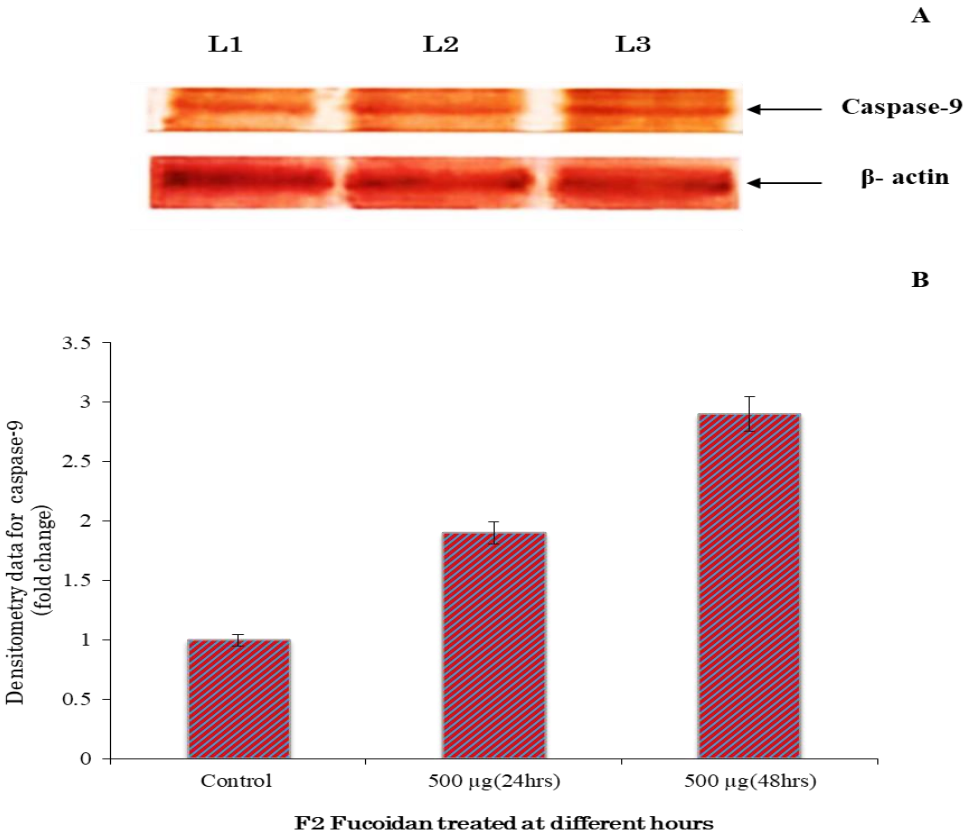

Fig. 16: (A) Representative Immunoblot expression of caspase-9 and β-actin to check equal protein of Control and F2-fucoidan treated cells.L1- Control MCF-7 cells, L2- Cells treated with 500 µg/ml of F2-fucoidan at 24 h, L3- Cells treated with 500 µg/ml of F2-fucoidan at 48 h. (B) Densitometry analysis of p53 western blotting. Densitometry data present as "fold change" as compared with control. Results are expressed as mean ± SD* $p<0.05$ vs control.

These results demonstrated that F2-fucoidan-treated MCF-7 cells are susceptible to apoptosis produced by a caspase-mediated mechanism. In the preceding research, the dose-dependent activity of *Cladosiphon okamuranus* on dose-dependent MCF-7 cells has been suggestive of highly antiproliferative and significantly cytotoxic. In addition to different apoptotic triggers, effector caspases including caspase-3, 8, and 9 result in DNA splitting. The Caspase-3 defect in MCF-7 cells is a caspase executor willing to cleave PARP (Germain *et al*., 1999; Liang *et al*., 2001; Chakraborti *et al*., 2004).

Conclusion

The present study demonstrates that sulfated polysaccharides with three proportions, F1, F2 and F3 were obtained from *C. implexa* J Agardh's thallus by extraction with 0.1M HCl followed by purification using DEAE cellulose quick flow anion-exchange chromatography. The fractions are assessed for their functions on antioxidant and anticancer by *in vitro* analysis. It was found that all the three fucoidan fractions expressed remarkable antioxidant activity and have order of F2 > F1 >F3. The increased sulfate concentration of the F2 fraction is connected to its stronger antioxidant activity. Both MTT and LDH testing offered further detail on their cytotoxicity of the F2 fraction. Both assays confirmed comparable amounts of cytotoxicity on MCF-7 cells with concentration dependent activity. Phase contrast microscopy morphological examination of fucoidan-treated MCF-7 cells shows that monolayer rupture at the end of 48 h caused cell death. Fluorescence microscopic observation by DAPI staining confirmed typical apoptotic elements in the MCF-7 cells that could be chromatin condensation,

nuclear fragmentation and apoptotic. Using flow cytometry to analyze the cell cycle, it was shown that F2-fucoidane possesses a number of antiproliferative properties against cancer cells. All the studies confirmed that the cellular death was time and dose dependent. The F2-fucoidan-induced DNA fragmentation in the cell line MCF-7 was observed and these findings indicated that F2-fucoidan was induced by apoptosis. Western blot research demonstrated that F2-fucoidan-treated MCF-7 cells are susceptible to and activated by a caspase-mediated pathway that result in apoptosis. Further studies are in progress on understanding the polysaccharide structure including glycoside linkage configuration, position of glucose connections, monosaccharide sequence, branch position and its relationship with biological function, which will help to develop potential anti-cancer drugs. Additional analysis of utilizing the extracted sulfated polysaccharides for *in vivo* model is undergoing.

Ethical Statement

Not applicable for this study.

Author Contributions

S.V., *S.U.M.R.*, *M.D.*, *S.M.*: Conception and design of the experiments; *M.D.*, *S.M.*, *R.A.D.*, *A.N.*: Execution of the experiments; *A.N.*, *J.G.*, *F.M.*: Data analysis; *S.U.M.R.*, *M.D.*, *S.M.*, *R.A.D.*: Manuscript writing; *J.G.*, *S.M.*: Manuscript editing. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The University Subvention Commission (UGC-CPEPA), New Delhi, India, provided the writers with monetary assistance, for which they are very appreciative. The authors have great recognition for the laboratory facilities given by the former Director Prof. R. Rengasamy, Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai, TamilNadu, India. The author Dr. S. U. Mohammed Riyaz also thanks the Management & the Principal of Islamiah College (Autonomous), Vaniyambadi, for providing necessary facilities in Animal Cell culture laboratory to carry out cell line studies and use of Inverted Fluorescence Phase Contrast Microscope for staining studies.

Conflict of Interest

The authors declare no conflicts of interest.

References

Berteau O and Mulloy B. (2003) Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. Glycobiology 13: R29–R40.

Bjornsson S. (1993) Size-dependent separation of proteoglycans by electrophoresis in gels of pure agarose. Analytical Biochem. 210: 292-298.

Campo VL, Kawano DF, Braz da Silva D and Carvalho I. (2009) Carrageenans: Biological properties, chemical modifications and structural analysis – A review. Carbohydrate Polymers 77: 167-180.

Chakraborti S, Michael JR and Chakraborti T. (2004) Role of an aprotinin-sensitive protease in protein kinase C a-mediated activation of cytosolic phospholipase A_2 by calcium ionophore (A23187) in pulmonary endothelium. Cell Signal 16: 751–762.

Chan TA, Hermeking H, Lengauer C, Kinzler KW and Vogelstein B. (1999) 14-3-3 α is required to prevent mitotic catastrophe after DNA damage. Nature 1: 616-620.

Chia YY, Kanthimathi MS, Khoo KS, Rajarajeswaran J, Cheng HM and Yap WS. (2015) Antioxidant and Cytotoxic activities of three species of tropical seaweeds. BMC Complem Altern Med. 15: 339.

Conn HJ. (1966) Biological stains. The Williams and Willams Co., Sigma Chemical Company, USA.

Costa LS, Fidelis GP, Cordeiro SL, Oliveira RM, Sabry DA, Câmara RBG, Nobre LTDB, Costa MSSP, Almeida-Lima J, Farias EHC, Leite EL and Rocha HAO. (2010) Biological activities of sulfated polysaccharides from tropical seaweeds. Biomed Pharmacother. 64: 21-28.

Di X, Chan KKC, Leung HW and Huie CW. (2003) Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high performance thin-layer chromatography. J Chromatogr A. 1018: 85-95.

Dias PF, Siqueira JM, Vendruscolo LF, de Jesus Neiva T, Gagliardi AR and Maraschin A. (2005) Antiangiogenic and antitumoral properties of a polysaccharide isolated from the seaweed *Sargassum*

stenophyllum. Cancer Chemother Pharmacol. 56: 436-446.

Dodgson KS and Price RG. (1962) A note on the determination of the ester sulphate content of SPs. Biochem J. 84: 106-110.

Duan, XJ, Zhang WW and Li XM. (2006) Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. Food Chemistry 95: 37-43.

Duarte MER, Cardoso MA, Noseda MD and Cerezo AS. (2001) Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. Carbohydrate Res. 333: 281-293.

DuBois M, Gilles KA, Hamilton JK, Rebers PA and Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem. 28: 350-356.

Ermakova S, Sokolova R, Kim SM, Um BH, Isakov V and Zvyagintseva T. (2011) Fucoidans from brown seaweeds *Sargassum hornery*, *Ecklonia cava*, *Costaria costata*: structural characteristics and anticancer activity. Appl Biochem Biotechnol. 164: 841-850.

Farias EHC, Pomin VH, Valente AP, Nader HB, Rocha HAO and Mourão PAS. (2008) A preponderantly 4-sulfated, 3-linked galactan from the green alga *Codium isthmocladum*. Glycobiology 18: 250-259.

Filisetti-Cozzi MCCT and Carpita NC. (1991) Measurement of uronic acids without interference from neutral sugars. Anal Biochem. 197: 157-162.

Flores ML, Stortz CA, Rodriguez MC and Cerezo AS. (1997) Studies on the skeletal cell wall and cuticle of the cystocarpic stage of the red seaweeds *Iridaea undulosa* Bory. Bot Mar. 40: 411-419.

Fouad Abdel-FA, Magdel-Din Hussein M and Mohamed Salem H. (1974) Studies of the purification and some properties of sargassan, a sulphated heteropoly-saccharide from *Sargassum linifolium*. Carbohydr Res. 33: 9-17.

Germain M, Affar EB, Amours DD, Dixit VM, Salvesen GS and Poirier GG. (1999) Cleavage of automodified poly (ADP-ribose) polymerase during apoptosis. J Biol Chem. 274: 28379-28384.

Han NS and Robyt JF. (1998) Separation and detection of sugars and alditols on thin layer chromatograms. Carbohydr Res. 313:135-137.

Haruki T, Kishi K and Zimmerman J. (1999) The importance of orofacial myofunctional therapy in pedatric dentistry: Reports of two cases. J Dent Child. 84: 103-109.

Johansen DA. (1940) Plant Microtechnique. McGraw-Hill, New York, pp. 523.

Keqi Wang HY, Zhou C, Liu J and Zeng X. (2008) Purification, antitumor and antioxidant activities *in vitro* of polysaccharides from the brown seaweed *Sargassum pallidum*. Food Chem. 111: 428-432.

King J. (1965) The transferases alanine and aspartate tranaminases. In: Practical Clinical Enzymology, (ed.) Van D., Nostrand Company Limited, London, pp. 121-138.

Koyanagi S, Tanigawa N, Nakagawa H, Soeda S and Shimeno H. (2003) Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. Biochem Pharmacol. 65: 173-179.

Kwon Kim S and Wijesekara I. (2010) Development and biological activities of marine-derived bioactive peptides: A review. J Functional Foods 2: 1-9.

Kylin H. (1913) Biochemistry of sea algae. H. Z. Physiol. Chem. 83:171-197.

Li B, Lu F, Wei X and Zhao R. (2008) Fucoidan: Structure and bioactivity. Molecules 13: 1671-1659.

Liang Y, Yan C and Schor NF. (2001) Apoptosis in the absence of caspase 3. Oncogene 45: 6570-6578.

Luo AX, He XJ, Zhou SD, Fan YJ, Luo AS and Chun Z. (2010) Purification, composition analysis and antioxidant activity of the polysaccharides from *Dendrobium nobile* Lindl. Carbohydrate Polymers 79:1014- 1019.

Mak W, Wang SK, Liu T, Hamid N, Li Y, Lu J and White WL. (2014) Anti-proliferation potential and content of fucoidan extracted from sporophyll of New Zealand *Undaria pinnatifida*, Front. Nutr. 1: 9.

Matou S, Helley D, Chabut D, Bros A and Fischer AM. (2002) Effect of fucoidan on fibroblast growth factor-2-induced angiogenesis *in vitro*. Thromb Res. 106: 213–21.

McCully ME. (1966) Histochemical studies on the genus *Fucus*. Light microscopy of the vegetative plant. Protoplasma 62: 287-305.

Miyamoto YY, Yamasaki M, Tachibana H and Yamada K. (2009) Fucoidan induces apoptosis through activation of caspase-8 on human breast cancer MCF-7 cells. J Agricult Food Chem. 57: 8677–8682.

Parker BC and Diboll AG. (1966) Alcian stains for histochemical localizations of acid and sulphated polysaccharide in algae. Phycologia 6: 37-46.

Piñeiro D, Elena Martín M, Guerra N, Salinas M and González VM. (2007) Calpain inhibition stimulates caspase-dependent apoptosis induced by taxol in NIH3T3 cells. Exp Cell Res. 313: 369-379.

Ponce NM, Pujol CA, Damonte EB, Flores ML and Stortz CA. (2003) Fucoidans from the brown seaweed *Adenocystis utricularis*: extraction methods, antiviral

activity and structural studies. Carbohydr Res. 338: 153-165.

Qiu X, Amarasekara A and Doctor V. (2006) Effect of oversulfation on the chemical and biological properties of fucoidan. Carbohydrate Polymers 63: 224-228.

Ramakrishnan G, Jagan D, Kamaraj S, Anandakumar P and Devaki T. (2009) Silymarin attenuated mast cell recruitment thereby decrease the expressions of MMPS-2 and 9 in rat liver carcinogenesis. Invest New drugs 27: 233-240.

Rashedy SH, Abd El Hafez MSM , Dar AM, Cotas J and Leonel P. (2021) Evaluation and characterization of alginate extracted from brown seaweed collected in the Red Sea. Appl Sci. 11: 6290-6307.

Robert KM, David AB, Kathleen MB, Peter JK, Victor WR and Anthony Weil P. (2009) Harper illustrated biochemistry. McGraw-Hill Companies, Inc.

Rupérez PP and Saura-Calixto F. (2001) Dietary fibre and physicochemical properties of edible Spanish seaweeds. Eur J Food Res Technol. 212: 349-354.

Sasaki T, Kawai K, Saijo-Kurit K and Ohno T. (1992) Detergent cytotoxicity: simplified assay of cytolysis by measuring LDH activity. Toxicol In Vitro 6: 451-457.

Shyamala V and Thangaraju N. (2014) Extraction of sodium alginate from selected seaweeds and their physiochemical and biochemical properties. Int J Innov Res Sci Engineer Technol. 3: 10998-11003.

Silchenko AS, Rasin AB, Zueva AO, Kusaykin MI, Zvyagintseva TN, Rubtsov NK and Ermakova SP. (2021) Discovery of a fucoidan endo-4O-sulfatase: Regioselective 4O-desulfation of fucoidans and its effect on anticancer activity *in vitro*. Carbohydrate Polymers 271: 118449.

Spector DL, Goldman RD and Leinwand LA. (1998) Cells: a laboratory manual. Cold Spring Harbor Laboratory Press. ISBN 9780879695224.

Sukirtha R, Priyanka K, Antony JJ, Kamlakkannan S, Thangam R, Gunasekaran P, Krishnan M and Achiraman S. (2012) Cytotoxic effect of green synthesized silver nanoparticles using *Melia azadirachta* against *in vitro* HeLa cell lines and lymphoma mice model. Proc Biochem. 47: 273-279.

Sundarraj S, Thangam R, Sreevani V, Kaveri K, Gunasekaran P and Kannan D. (2012) γ- Sitosterol from *Acacia nilotica* L. induces G2/M cell cycle arrest and apoptosis through c-Myc suppression in MCF-7 and A549 cells. J Ethnopharmacol. 141: 803–809.

Suresh V, Anbazhagan C, Thangam R, Senthilkumar D, Senthilkuma N, Kannan S, Rengasamy R and Palani P. (2013) Stabilization of mitochondrial and microsomal function of fucoidan from *Sargassum plagiophyllum* in diethylnitrosamine induced hepatocarcinogenesis. Carbohydrate Polymers 92: 1377-1385.

Wijesekara I, Pangestuti R and Kwon Kim S. (2011) Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. Carbohydrate Polymers 84 : 14-21.

Xue CH, Fang Y, Lin H, Chen LZJ and Deng DL. (2001) Chemical characters and antioxidative properties of sulfated polysaccharides from *Laminaria japonica*. J Appl. Phycol. 13: 67-70.

Yang C, Chung D, Shin I, Lee H, Kim J, Lee Y and You S. (2008) Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida.* Int J Biol Macromolec. 43: 433-437.

You SD, Yang C, Lee HY and Lee BY. (2010) Molecular characteristics of partially hydrolyzed fucoidans from sporophyll of *Undaria pinnatifida* and their *in vitro* anti-cancer activity. Food Chem. 119: 554-559.

Zemani Benisvy F, Galy D, Lokajczyk FI, Colliec AJS and Uzan G. (2005) Low-molecular weight fucoidan enhances the proangiogenic phenotype of endothelial progenitor cells. Biochem. Pharmacol. 70: 1167-1175.