International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (3): 974-983
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Structural Elucidation and Efficacy Comparison of Crude Powder of Selected Brown Seaweed and Isolated Sulphated Polysaccharide Towards Anti-Inflammation by Protein Denaturation Method

S. Deepa[1*], D. Velmurugan[2], K. Sujatha[1] and K. Sathesh Kumar[1]
[1*&1]Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research (SRIHER) - DU, Porur, Chennai.
[2]Centre of Advanced Study in Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai.

Received: 21 Mar 2019 / Accepted: 23 Apr 2019 / Published online: 1 Jul 2019
***Corresponding Author Email:** deepaselvarajs@gmail.com

Abstract
Brown algae consists of Fucoidan, a sulphated polysaccharide as its main constituents especially in its extracellular matrix. It has various biological activities, like immunomodulatory, anti- tumour, anti-inflammatory, anti-coagulant, etc. Prescribed methods based on the reference articles were followed for Extraction followed by Purification of Fucoidan from *Turbinaria ornate.* This study is to determine the anti-inflammatory potency of the isolated fucoidan from the selected brown seaweed, after proper purification and characterization using IR, NMR and GC-MS collected from Mandabam area, Ramanathapuram District and compared with that of the crude powder of the same selected seaweed. The results revealed that the isolated and purified Fucoidan has potential anti-inflammatory activity than the crude *T.ornate* powder using protein denaturation method.

Keywords
Brown Seaweed, Sulphated Polysaccharide, Cell line.

*****

1. INTRODUCTION:
Emergent substantiation implies that systemic inflammation has probability of enhanced risk of many chronic diseases [1, 2]. Inflammatory mechanism may include activation of macrophages and T lymphocytes, the release of proinflammatory mediators, like tumor necrosis factor- (TNF-) α, IL-6, interleukin- (IL-) 1, prostaglandin E2 (PGE2) and nitric oxide (NO), augment the inflammatory activity [3]. Innate immune responses get effectively promoted because of the appropriate production of these mediators; but still extreme inflammation leads to sepsis, and even life threatening [4]. Brown seaweed consists of a cell-wall matrix polysaccharide which is

composed of ester sulfate and α-l-fucose and also traces of d-Glc, d-GlcA, d-Gal, d-Man, d-Xyl, and acetic acid [5]. The fucoidans structure differs among various brown seaweed has influence on the biological activity [6,7,8] because of their difference in the linkage pattern, ester sulfate content, monosaccharide moieties present and their corresponding molecular weight [9]**.**This is because of the difference in ecophsiological parameters [7] It is reported to possess various biological activity including anti-coagulant [10], anti-viral [11,12], anti-tumour [13], Immunostimulant [14], Anti-bacterial [15], Anti-coagulant [16,17], Anti-inflammatory [18] and Anti-oxidant properties [16] with the remarkable absence of adverse effects. Copious effort in to determine the structure of this sulfated polysaccharide in the past few decades using IR, NMR [19,20,21], Mass [20,22] was taken. However adequate understanding about their binding ability with the targeted proteins and its structure is essential [23]. This study is to determine the anti-inflammatory potency of the isolated fucoidan from the selected brown seaweed, after proper purification and characterization using IR, NMR and GC-MS collected from Mandabam area, Ramanathapuram District.

2. MATERIALS AND METHODS:

2.1 Collection: collection of *Turbinaria ornata* was done from intertidal regions of Mandabam area (Ramanathapuram District) of the Gulf of Mannar, Southern east coastal of India. This area is reported to be the world's richest regions from the marine biodiversity perspective [24].

2.2 Chemicals: Acetone: Methanol (7:3, v/v),0.5M HCl, Absolute Ethanol, 0.1M Sodium Phosphate Buffer pH 7.2, Dialysis Tube, 0.2, 0.7 and 1.5M NaCl solution and Calcium chloride.

2.3 Extraction of Fucoidan from *Turbinaria ornata*: Seaweed biomass was thoroughly washed with seawater to remove debris instantly and then with tap water and finally with distilled water. Shade dried and grind into pieces of 1 mm. Algal powder approximately 100 g was weighed and drenched in the solvent system of Acetone-Methanol at the ratio of 7:3, v/v for around 2 days at 200 rpm in orbital shaker. The above said soaking procedure was continual twofold to make sure the whole discolouration and defatting of waterless biomass which was shade dried and powdered. It is then extracted in 0.5 M of HCl, 1 L with stirring rate at 200 rpm constantly at 90°C water bath for about 4 h. The pellet was re-extracted as above and the supernatants were pooled. The supernatant was precipitated with 1:1 volume of 4% $CaCl_2$for alginate removal at 4°C for overnight. The compiled supernatant solutions was precipitated using two volume of absolute ethanol at the ratio of 1:2 (v:v) after keeping it at overnight at 4ºC. The collected precipitate was made to dissolve in aqueous medium and dialysed using MW CO 14,000 membrane for 2 days at 4ºC. It is then freeze dried and stored in air tight container.

2.4 Purification of Fucoidan: The purification of the freeze dried crude polysaccharides was done by dissolving in 0.1 M of pH 7.2 sodium phosphate buffer and filled to Q-Sepharose fast flow column with the dimensions of 4×25 cm. Using 0.1 N Sodium Phosphate buffer stepwise elution was done with 0.2, 0.7, and1.5 M sodium chloride solutions and the flow rate is fixed to 60 mL/h. The eluent was collected in various tubes each with 5 ml was examined for carbohydrate content using the standard, Fucose. Hence the final product, Carbohydrates thus obtained with various fractions of polysaccharide was assayed using the procedure reported in the article [25]. Using water, it is dialyzed and then lyophilized for future study.

2.5 Chemical analysis: To estimate fucose content, the standard Phenol-Sulphuric acid method [25] was used. L-Fucose as standard and Similarly for estimating Sulphate content [26] using Sodium sulfate as standard.

2.6 Fourier Transform Infrared Spectroscopy (FTIR): FTIR spectrophotometer (Thermo Scientific Nicolet IS10, USA) in ATR mode was used to measure FTIR spectra of the isolated Sulphated polysaccharide. The resolution was of 4 cm−1 and the range of wavelength is 800–4000 cm−1 wave numbers.

2.7 NMR analysis: The number of protons present in the isolated compound was predicted by 1H NMR experiments [27] using NMR spectrometer Bruker Biospin Advance 400. The Isolated and purified sulphated polysaccharide was liquefied with 0.5 ml Deuterium oxide. The 1H frequency was maintained to ma¼ 400.13 MHz at 298 K with a broad band of 5-mm the equipment has inverse probe head shielded with z-gradient and XWINNMR software version 3.5. Internal reference used was TMS. One-dimensional 1H spectra were acquired was by one pulse sequence.

2.8 Analysis of monosaccharide compositions. 10 mg of the Isolated sulfated polysaccharide was accurately weighed and dissolved using of trifluoroacetic acid, 2mol/l. It is kept aside at 100°C for 12 hours so that the polysaccharides were entirely hydrolyzed into monosaccharides. After the completion of the hydrolysis, co-distillation with

methanol was done to eliminate excess acid. Sugar moieties are converted into alditol acetates by treating 2ml of pyridine and 1.5 ml of acetic anhydride. Using Gas Chromatography equipmencolumn of t with SE-54 fused silica capillary (320 µm × 50 m) (HP6890; Agilent Technologies Co. Ltd., USA) armed with FI detector Gas Chromatography was carried out. The operation condition includes: 200ml/min- air, 1.5 ml/min - N_2 and H_2 each, 250°C – Injection temperature and Detector temperature each; 212°C Column temperature. Monosaccharide identification was done using reference standards of Xylose, Galactose, Mannose and Glucose [28].

2.9 Protein Denaturation Method: In the present study the protein denaturation bioassay [29] is the *in-vitro* method chosen to assess anti-inflammatory potency of the crude *Turbinaria ornata* seaweed and Isolated Sulphated polysaccharide. Tissue protein denaturation is the major cause of inflammation and Arthritis. Denaturation leads to Auto Antigen Production in few inflammatory condition *in-vivo.* Thus the compounds require to Agents that can hinder such protein denaturation is utilitarian for the ant-inflammatory drug development. 0.5 ml of the test solution consists of 0.45 ml of Bovine Serum Albumin (5% w/v aqueous solution) and 0.05 ml of test solution. Similarly, 0.5ml Test control solution with Bovine serum albumin 0.45 % consisting of 5% w/v aqueous solution and distilled water 0.05 ml. 0.5ml of product control is made up of distilled water 0.45ml and test solution 0.05ml. The standard solution was prepared with a mixture of Bovine serum albumin 0.45 ml which is 5% w/v aqueous solution and Diclofenac sodium 0.05 ml, a standard drug. The test solution of the algal extracts was prepared with the following concentrations viz., 50,100,250,500,1000,2000 in µg/ml and compared with that of the same concentrations of standard solution using Diclofenac Sodium. Finally, the entire solutions together were subjected to incubation at 37ºC for 3 mins. To the above solution 2.5 ml of phosphate buffer was added and cooled. Using UV – Visible spectrometer at 416 nm the absorbance was measured considering the control as 100% protein denaturation.

The following formula was used to calculate the percentage inhibition of protein denaturation

$$\text{Percentage inhibition} = \frac{[100 - (\text{Optical density of test solution} - \text{optical density of product control})]}{(\text{Optical density of test control})}$$

3. RESULTS AND DISCUSSION:

The practical yield of extracted fucoidan from *T. ornate* was 5.18 ± 0.52 %. The obtained fucoidan, composed of 60.9 ± 0.14 % fucose and 25.4± 0.38 % sulfate. **IR analysis:** In the study IR spectrum was mentioned as Fig 1. The IR band at 2930 cm^{-1} indicates the presence of C-H Stretching of C-6 group of Galactose and Fucose unit and of pyranose ring. C-O-S bending vibration of sulfate substituent present in the axial C-4 position was confirmed because of the presence of band at 836 cm^{-1}. The stronger band in the region of 1259-1051 cm $^{-1}$ was due to C-C and C-O stretching vibrations of pyramid ring as well as C-O-C stretches of the glycosidic bonds. The presence of asymmetric O=S=O stretching of sulfate esters with C-O-H, C-C and C-O vibrations are because of the band present at 1259 cm^{-1}. The three bands appeared in the region between 3600-1600cm^{-1}, with a broad band centered at 3454 cm^{-1} was consigned to Hydrogen bonded O-H stretching vibrations. **NMR analysis:** In the present study ^{1}H NMR spectrum was shown in Fig 2a-2c. The signal noted at 1.55 ppn shown the existence of alkyl proton in which Sulfonyl is attached. Signals between the region of 3.45 to 4.76 ppm entrusted to methoxy attached proton at H-4 position. The sharp absorptions in the ^{1}H NMR corresponds to the presence of (1-6)- β- D- linked Galacton at the ppm of 4.48 for H-1, 3.78 for H-5, 3.69 for H-3, 4.08/3.72 for H-6/H-6′ and 4.08 for H-4. The ^{1}H adsorption at ppm of 5.55, 5.33,5.26 corresponds to the presence of α- L- fucose terminal, 3-linked α- L-fucose and 3,4 distributions of α – L-fucose. Thus proposing the presence of 3 sulfated 4 linked and 4 sulfated 3-linked α-L- fucose.

By the same token the ^{1}H spectrum signals in terms of ppm at 5.20 is for H-1, 3.78 for H-2, 3.72 for H-3, 3.72 for H-4 and 1.19 for H-6 (Fig. 2). **GC-MS analysis:** The GC-MS analysis of the isolated and purified Fucoidan from the selected seaweed *T. ornate* signposted the presence of the monomers including Galactose, Mannose penta acetate, Glucose methyl glycoside tetra acetate, Diethyl (3,4,5-trimethoxybenzoyl) malonate, D- Mannose, Xylose, Z-E-2-Methyl-3,13-octadecadien-1-ol. The purified fucoidan was examined by GC-MS and the results disclosed the retention time of 16.08, 17.68, 19.17, 20.5, 9.87, 12.18 and 14.27 respectively. Fig 3a -3h. explicates the GC-MS analysis of purified Fucoidan.

Hence the 8 peaks along with their Retention times were shown in fig 3i. **Protein Denaturation Method:** The Crude powder of *Turbinaria ornata* and the isolated and purified Fucoidan were subjected to testing of its anti-inflammatory potency using protein Denaturation method using Diclofenac Sodium as standard. The purified Fucoidan exhibited respectable activity with utmost inhibition of about 86.60 % and crude *T.ornata* was about 79.08 % when paralled to standard Diclofenac Sodium at the same concentration for which the percentage inhibition was 98.3%. The results were presented in Tab 1 and Fig 4.

4. CONCLUSION:

The forte of biological activity of fucoidan depends on the fucoidan structure with various ratios of fucose and ester sulphate contents from *T. ornata* are now under investigation. The Current study result discloses that the reason for the better anti-inflammatory potency of the isolated fucoidan than that of the crude powder consisting of many constituents other than that of the fucoidan, may be because of the sulphate content and also when the fucoidan was isolated, the effect was enhanced because of the antagonistic effects of some other constituents in the crude. However, further detailed examination is required. In conclusion, the isolated fucoidan from *T. ornata* can be an option for anti-inflammatory agent thereby to control inflammation.

Figure 1: FT-IR spectrum of Fucoidan from *T.ornata.*

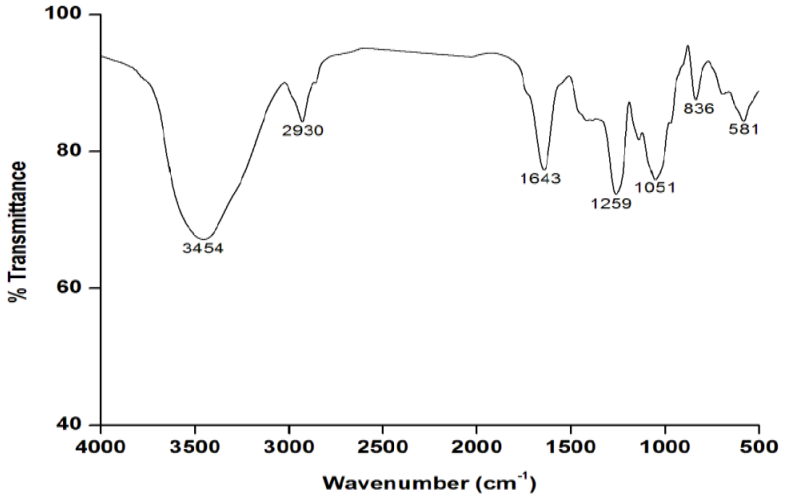

Figure: 2a NMR spectrum of Isolated Fucoidan

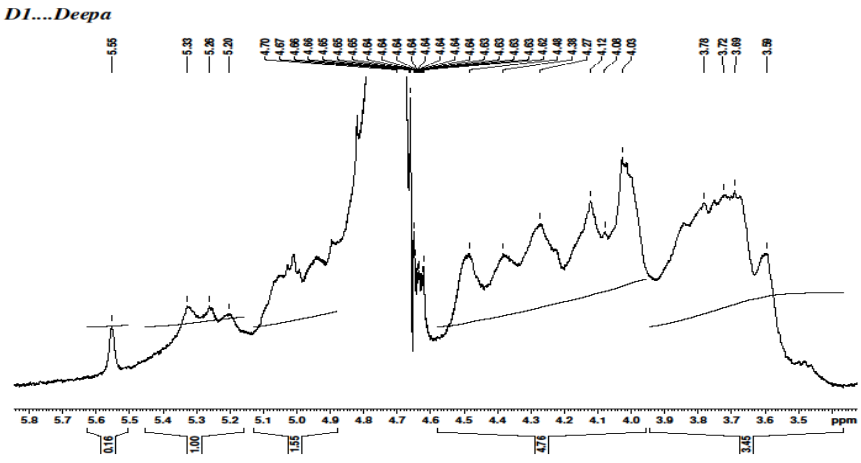

Figure: 2b NMR spectrum of Isolated Fucoidan

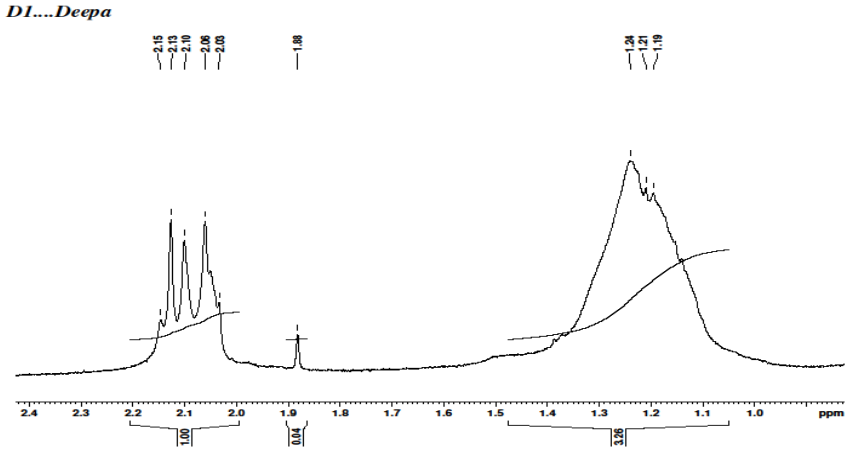

Figure: 2c NMR spectrum of Isolated Fucoidan

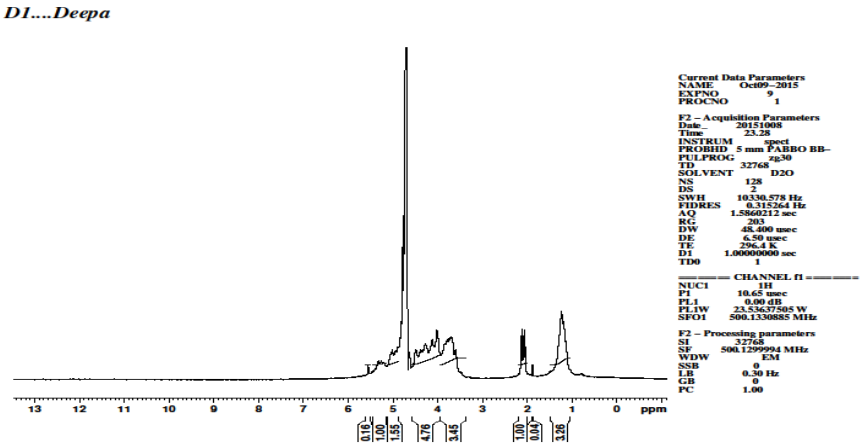

Figure: 3a GC-MS analysis of purified fucoidan for Galactose monomer

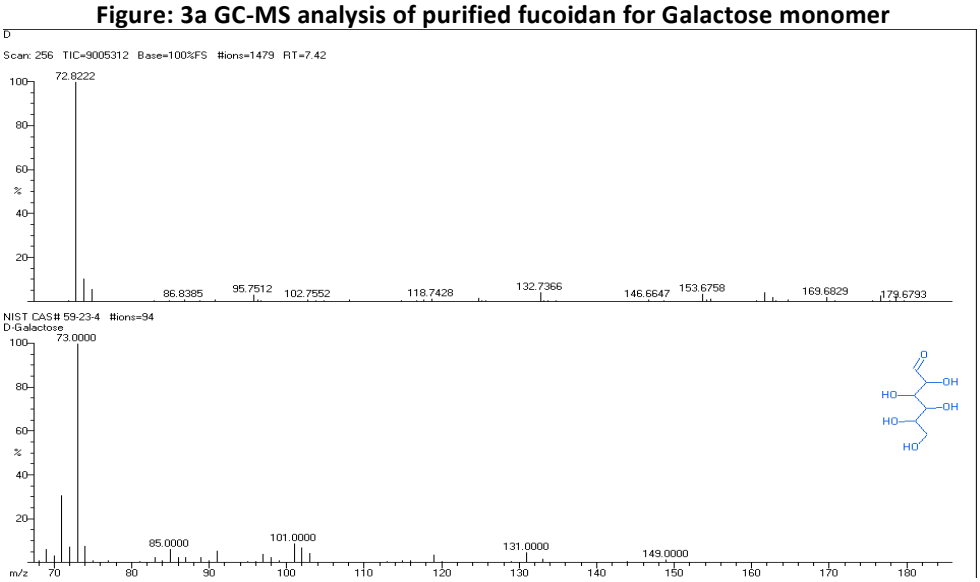

Figure: 3b GC-MS analysis of purified fucoidan for D mannose monomer

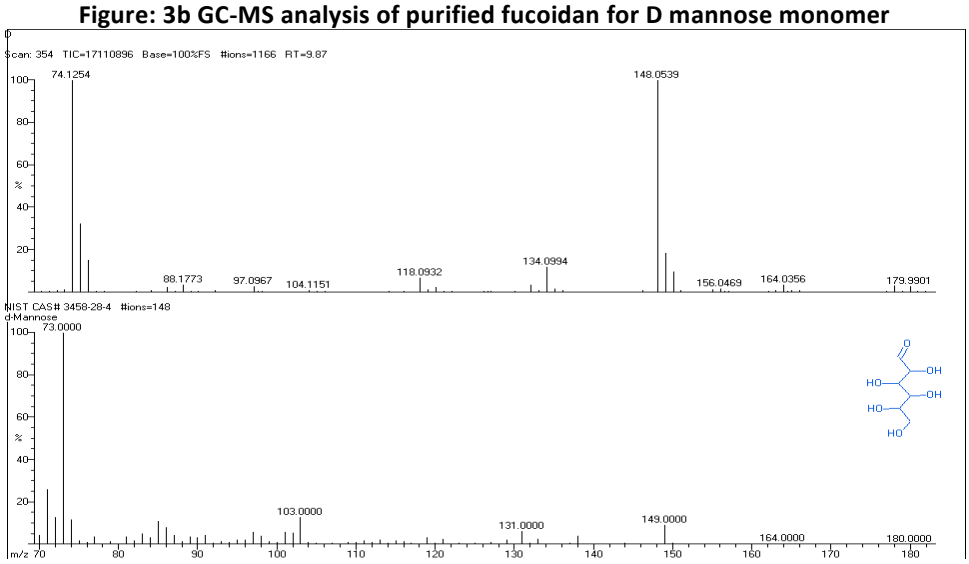

Figure: 3c GC-MS analysis of purified fucoidan for Xylose monomer

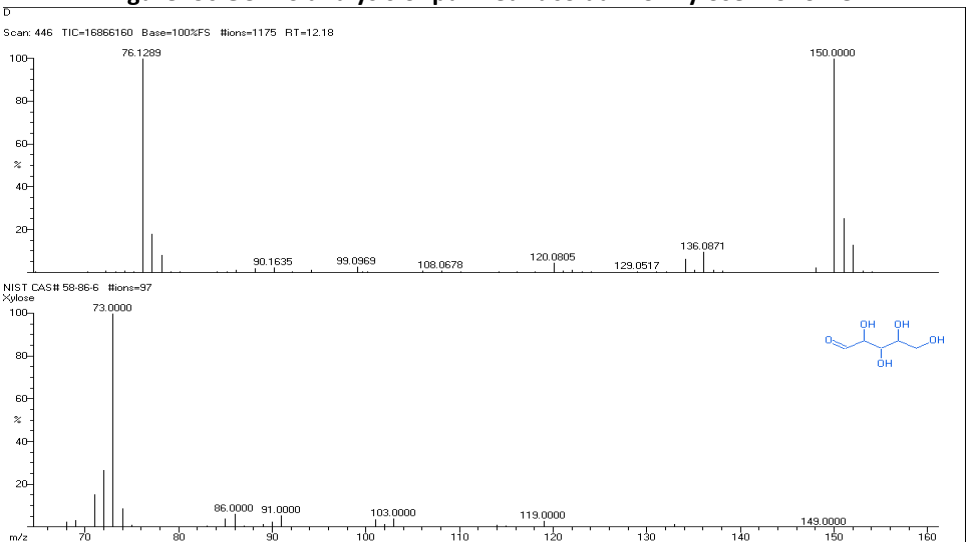

Figure: 3d GC-MS analysis of purified fucoidan for Z-E-2-Methyl-3,13-octadecadien-1-ol.

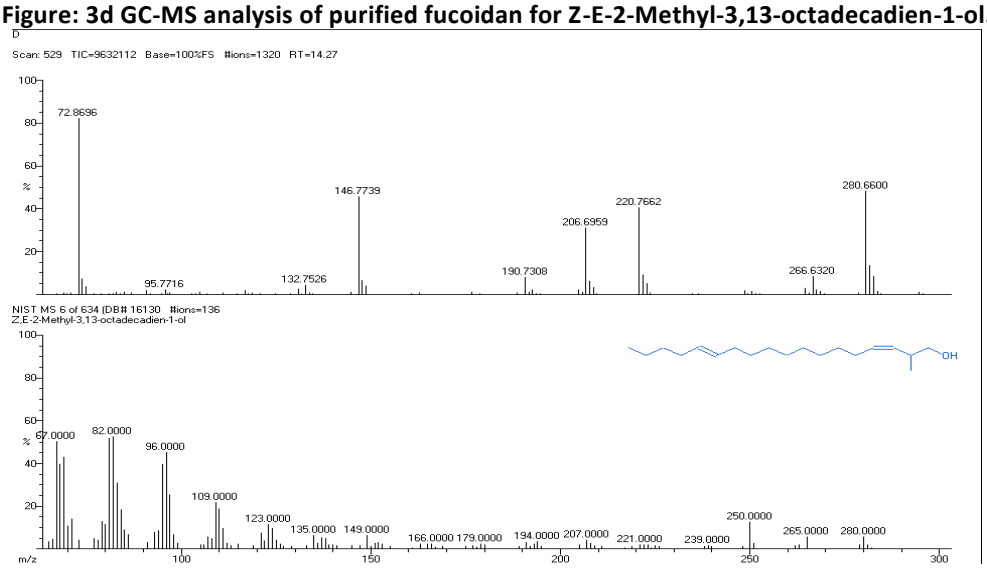

Figure: 3e GC-MS analysis of purified fucoidan for Galactose monomer

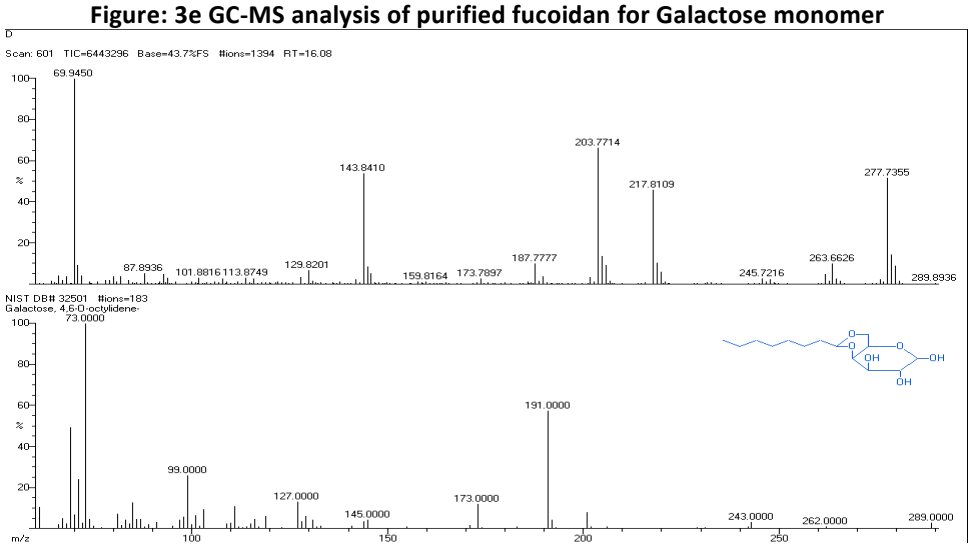

Figure: 3f GC-MS analysis of purified fucoidan for Mannose penta acetate monomer

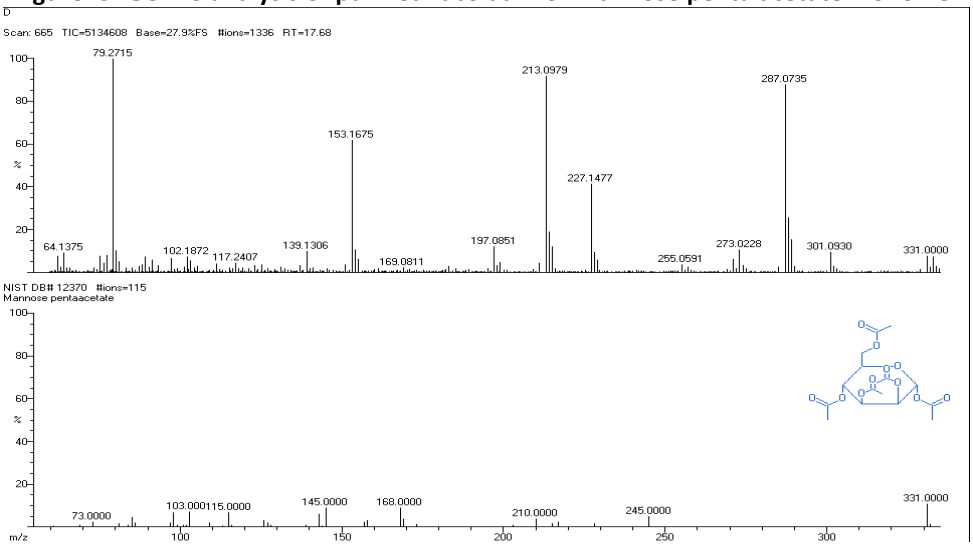

Figure: 3g GC-MS analysis of purified fucoidan for Glucose methyl glycoside tetra acetate monomer

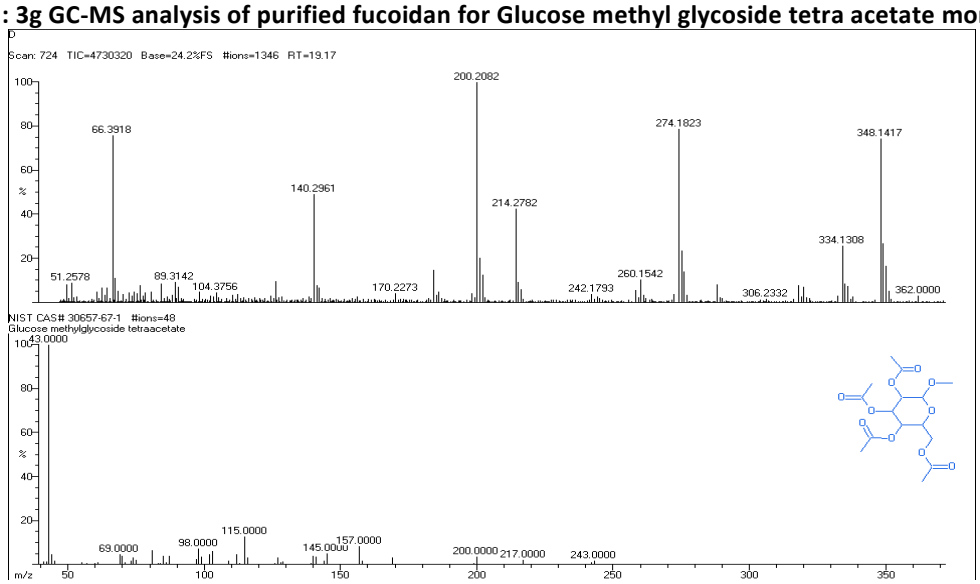

Figure: 3h GC-MS analysis of purified fucoidan for Diethyl (3,4,5- trimethoxybenzoyl) malonate monomer

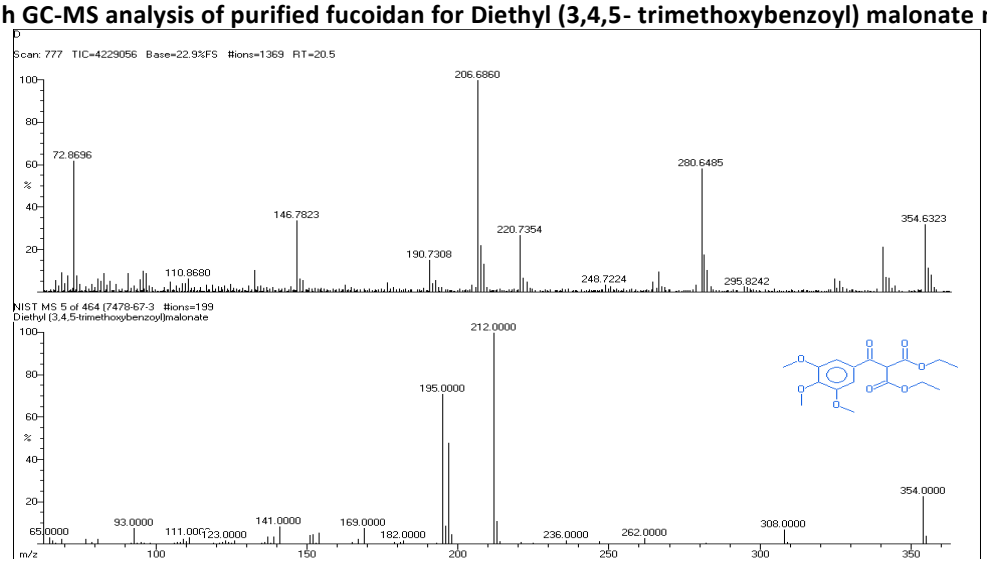

Figure: 3i GC-MS spectra of purified fucoidan.

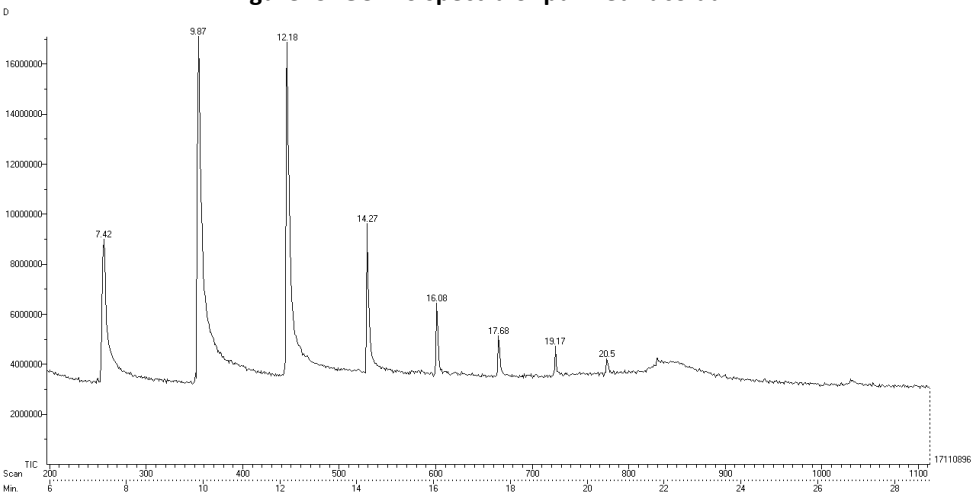

Figure: 4 Anti-inflammatory effect of Both Crude *Turbinaria ornata* powder and Isolated and Purified Fucoidan by Inhibition of Protein Denaturation

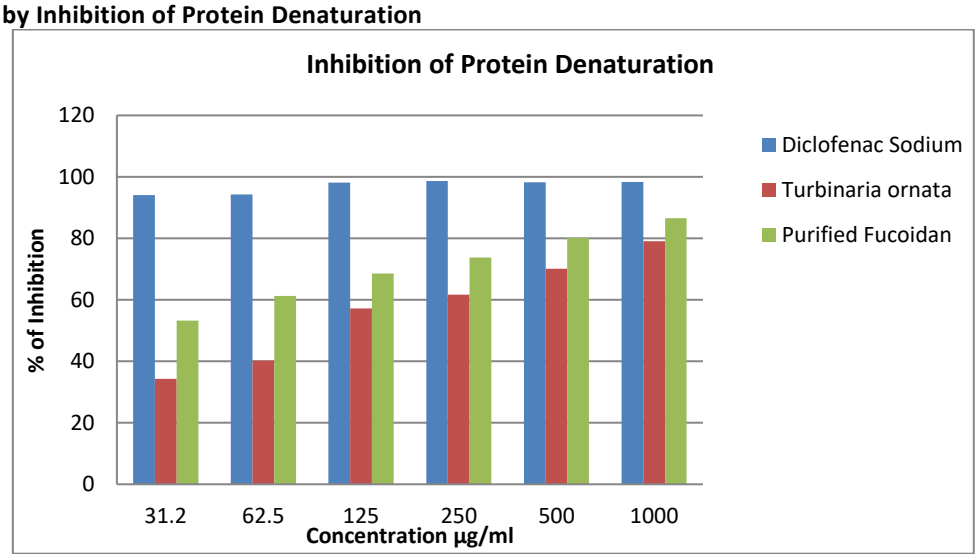

Table: 1 Anti-inflammatory effect of Both Crude *Turbinaria ornata* powder and Isolated and Purified Fucoidan by Protein Denaturation method

S.NO	CONCENTRATION (µg/ml)	% INHIBITION		
		DICLOFENAC SODIUM	*Turbinaria ornata* Crude powder	*Purified Fucoidan*
1	31.2	94.1	34.26	53.2
2	62.5	94.3	40.28	61.3
3	125	98.10	57.23	68.6
4	250	98.64	61.66	73.8
5	500	98.27	70.14	80.1
6	1000	98.3	79.08	86.6

REFERENCES:

1. Hong, Y.H., Weng, L.W., Chang, C.C., Hsu, H.F., Wang, C.P., Wang, S.W. and Houng, J.Y., 2014. Anti-inflammatory effects of Siegesbeckia orientalis ethanol extract in in vitro and in vivo models. *BioMed research international,* 2014.
2. Libby, P., 2006. Inflammation and cardiovascular disease mechanisms–. *The American journal of clinical nutrition,* 83(2), pp.456S-460S.
3. Lin, W.W. and Karin, M., 2007. A cytokine-mediated link between innate immunity, inflammation, and cancer. *The Journal of clinical investigation,* 117(5), pp.1175-1183.
4. Das, U.N., 2000. Critical advances in septicemia and septic shock. *Critical care,* 4(5), p.290.
5. Shiroma, R., KoniShi, T., Uechi, S. and TaKo, M., 2008. Structural study of fucoidan from the brown seaweed Hizikia fusiformis. *Food science and technology research,* 14(2), pp.176-182.
6. Costa, L.S., Fidelis, G.P., Cordeiro, S.L., Oliveira, R.M., Sabry, D.D.A., Câmara, R.B.G., Nobre, L.T.D.B., Costa, M.S.S.P., Almeida-Lima, J., Farias, E.H.C. and Leite, E.L., 2010. Biological activities of sulfated polysaccharides from tropical seaweeds. *Biomedicine & Pharmacothe rapy,* 64(1), pp.21-28.
7. Chollet, L., Sabourał, P., Chauvierre, C., Villemin, J.N., Letourneur, D. and Chaubet, F., 2016. Fucoidans in nanomedicine. *Marine drugs,* 14(8), p.145.
8. Ale, M.T., Mikkelsen, J.D. and Meyer, A.S., 2011. Important determinants for fucoidan bioactivity: A critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds. *Marine drugs,* 9(10), pp.2106-2130.
9. 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, 29R-40R
10. Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A.M. and Boisson-Vidal, C., 1999. Further data on the structure of brown seaweed fucans: relationships with anticoagulant activity. *Carbo hydrate Research,* 319(1-4), pp.154-165.
11. Baba, M., Snoeck, R., Pauwels, R. and De Clercq, E., 1988. Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. *Antimicrobial agents and chemotherapy*, *32*(11), pp.1742-1745.
12. Ponce, N.M., Pujol, C.A., Damonte, E.B., Flores, M.L. and Stortz, C.A., 2003. Fucoidans from the brown seaweed Adenocystis utricularis: extraction methods, antiviral activity and structural studies. *Carbohydrate Research*, 338(2), pp.153-165.
13. Itoh, H., Noda, H., Amano, H., Zhuaug, C., Mizuno, T. and Ito, H., 1993. Antitumor activity and immunological properties of marine algal polysaccharides, especially fucoidan, prepared from Sargassum thunbergii of Phaeophyceae. *Anticancer research,* 13(6A), pp.2045-2052.
14. Immanuel, G., Sivagnanavelmurugan, M., Marudhupandi, T., Radhakrishnan, S. and Palavesam, A., 2012. The effect of fucoidan from brown seaweed Sargassum wightii on WSSV resistance and immune activity in shrimp Penaeus monodon (Fab). *Fish & shellfish immunology,* 32(4), pp.551-564.
15. Vijayabaskar, P. and Shiyamala, V., 2011. Antibacterial activities of brown marine algae (Sargassum wightii and Turbinaria ornata) from the Gulf of Mannar Biosphere Reserve. *Advances in Biological Research,* 5(2), pp.99-102.
16. Arivuselvan, N., Radhiga, M. and Anantharaman, P., 2011. In vitro antioxidant and anticoagulant activities of sulphated polysaccharides from brown seaweed (Turbinaria ornata) (Turner) J. Agardh. *inflammation,* 14(15), p.16.
17. Deepak, P., Sowmiya, R., Balasubramani, G. and Perumal, P., 2017. Phytochemical profiling of Turbinaria ornata and its antioxidant and anti-proliferative effects. *Journal of Taibah University Medical Sciences*, *12*(4), pp.329-337.
18. Ananthi, S., Gayathri, V., Chandronitha, C., Lakshmi sundaram, R. and Vasanthi, H.R., 2011. Free radical scavenging and anti-inflammatory potential of a marine brown alga Turbinaria ornata (Turner) J. Agardh.

19. Chevolot, L., Mulloy, B., Ratiskol, J., Foucault, A. and Colliec-Jouault, S., 2001. A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae. *Carbohydrate Research,* 330(4), pp.529-535.
20. Daniel, R., Chevolot, L., Carrascal, M., Tissot, B., Mourão, P.A. and Abian, J., 2007. Electrospray ionization mass spectrometry of oligosaccharides derived from fucoidan of Ascophyllum nodosum. *Carbohydrate Research,* 342(6), pp.826-834.
21. Thanh, T.T.T., Tran, V.T.T., Yuguchi, Y., Bui, L.M. and Nguyen, T.T., 2013. Structure of fucoidan from brown seaweed Turbinaria ornata as studied by electrospray ionization mass spectrometry (ESIMS) and small angle X-ray scattering (SAXS) techniques. *Marine drugs, 11*(7), pp.2431-2443.
22. Bilan, M.I., Grachev, A.A., Ustuzhanina, N.E., Shashkov, A.S., Nifantiev, N.E. and Usov, A.I., 2002. Structure of a fucoidan from the brown seaweed Fucus evanescens C. Ag. *Carbohydrate research,* 337(8), pp.719-730.
23. Clément, M.J., Tissot, B., Chevolot, L., Adjadj, E., Du, Y., Curmi, P.A. and Daniel, R., 2010. NMR characterization and molecular modeling of fucoidan showing the importance of oligosaccharide branching in its anticomplementary activity. *Glycobiology,* 20(7), pp.883-894.
24. Neelamathi, E. and Kannan, R., 2015. Screening and characterization of bioactive compounds of Turbinaria ornata from the gulf of Mannar, India. *Screening*, 2(11).
25. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T. and Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Analytical chemistry,* 28(3), pp.350-356.
26. Hou, Y., Wang, J., Jin, W., Zhang, H. and Zhang, Q., 2012. Degradation of Laminaria japonica fucoidan by hydrogen peroxide and antioxidant activities of the degradation products of different molecular weights. *Carbohydrate Polymers,* 87(1), pp.153-159.
27. Marudhupandi, T. and Kumar, T.T.A., 2013. Effect of fucoidan from Turbinaria ornata against marine ornamental fish pathogens. *Journal of Coastal Life Medicine,* 1(4), pp.282-286.
28. Fu, H.N., Zhao, X., Yu, G.L., Chen, E. and Wen, S., 2008. Comparison of four chromatographic methods for monosaccharide composition analysis of Dunaliella salina polysaccharide. *Chinese Journal of Marine Drugs,* 27(4), pp.30-34.