BIOLOGICAL POTENTIAL OF FUCOIDAN FROM SARGASSUM TENERRIMUM

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Abstract: The present study has been undertaken to investigate the biological properties of fucoidan extracted from *Sargassum tenerrimum.* The fucoidan was extracted by hot water extraction process followed by ethanol precipitation. The biochemical composition and biological potential of extracted fucoidan was evaluated. The fucoidan showed significant biological activities in relation with antioxidant (91.21%), anti-inflammatory (92.60%) and anticoagulant (> 1000s prolongation).

Index Terms **– Fucoidan, antioxidant, anticoagulant, anti-inflammatory.**

I. INTRODUCTION

Recently, concerns have been shifted towards the use of renewable natural resources. This shift diverted the focus from terrestrial resources towards the study of marine resources. Among the reserves of huge marine resources lay the seaweeds along the edges of the oceans. Seaweeds are multicellular, macroscopic, marine algae which utilize sunlight as an energy source to convert carbon dioxide and water into carbohydrates .They fall into the diverse category of photosynthetic organisms which have evolved into around 36,000 known species. They are classified according to their ecology, habitat, size, pigments, polysaccharides, cell culture, composition and morphology. The seaweeds are further grouped into three categories; red seaweed (Rhodophyta) with 6500 known species, brown seaweed (Phaeophyta) with 1800 known species and green seaweed (Chlorophyta) with approximately 1500 known species. They are being utilized as food, fertilizers and phycocolloids throughout the world (Talha Bin Saleem Ahmad, 2015).

Brown seaweeds are found along the coastal region of the world and prefer growing in cold water. They are able to grow under water depths of around 30-50 meters but they are mostly present in the intertidal and upper sub littoral zones of the oceans. Brown seaweeds generally absorbs green light of medium wavelengths to perform their photosynthesis. One of the brown seaweed namely *S. tenerrimum* has a high marine ecological importance. The alga has a high biomass production, greater dominance and has an important ecological distribution. The kelp forest *S. tenerrimum* in the ocean provides food and shelter for thousands of fish, invertebrates and marine mammal species. *S. tenerrimum* is widely distributed in the coastal regions of Indian Ocean Islands Andaman Islands (Silva, Basson & Moe, 1996), Nicobar Islands (Silva, Basson & Moe 1996).

S. tenerrimum possesses bioactive polysaccharide namely "fucoidan" that has recently gained large attention because of its huge applications in health sector. It has been reported that Fucoidan has anticancer, anticoagulant, antioxidant and antiviral properties (Li et al., 2008). Fucoidan is a family of sulfated fucose rich in polysaccharide that is present in the extracellular matrix of brown seaweeds (Albuquerque et al., 2004). In recent years, sulfated polysaccharides from marine algae have been demonstrated to have many biological activities such as anticoagulant (Shanmugam and Mody, 2000), antioxidant (Ruperez et al., 2002), and antiviral (Duarte et al., 2001). The fucoidan bioactivity is dependent upon two key factors: The method of extraction; and the seaweed species from which it has been derived. These two factors are the ultimate determinants of efficacy. The extraction procedures greatly influence the stability and functionality of the fucoidan.

Anticoagulant activities are the most widely studied properties of sulfated polysaccharides. Anticoagulant activity of sulfated polysaccharides has been identified from several brown seaweeds such as *Padina gymnospora* (Silva et al., 2005), *Spatoglossum schroederi* (Leite et al., 1998), and the green seaweed *Codium cylindricum* (Matsubara et al., 2003). Specific structural features in sulfated polysaccharides are required for anticoagulant activity, especially negatively charged sulfated clusters to ensure interaction with cationic proteins. Anticoagulant activities are among the most widely studied properties of marine algae (Farias et al., 2000). The anticoagulant activity of sulfated polysaccharides, especially fucoidan highly depends on the sulfate content and its molecular size (Nishino et al., 1989).

Antioxidants neutralize potentially harmful reactive free radicals in body cells, and may reduce potential mutations and thereby help prevent cancer and heart disease. Consequently, cellular and extracellular macromolecules such as proteins, lipids, and nucleic acids can suffer oxidative damage, causing tissue

injury (Halliwell & Gutteridge, 1989). A requirement for endogenous antioxidant capacity in algae is implicit, due to the fact that algae, as intertidal organisms, require protection against UV irradiation (Swanson and Druehl, 2002) and the effects of desiccations from daily tidal fluctuations (Burritt et al., 2002). The presence of sulfate groups in seaweed polysaccharides is responsible for numerous types of biological activities, such as antioxidant activities (Qi et al., 2005; Wang et al., 2009).

Inflammation is the normal physiological and immune response to tissue injury. Increased blood supply, enhanced vascular permeability and migration of immune cells occur at damaged sites .The inflammatory process is a protective response that occurs in response to trauma, infection, tissue injury or noxious stimuli. It can be identified by tumor (swelling); Robr (redness) Calor (heat) and Dolar (pain) (Sunita Verma, 2016). Seaweed is one of the potential objects for the extraction of anti-inflammatory agents. Seaweeds have a rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential. The symptoms of inflammation include pain, redness, heat and swelling. When there is injury to any part of the human body, the arterioles in the encircling tissue dilate that result in raised blood circulation in the affected region causing redness.

Inflammation is either acute or chronic inflammation. The acute inflammation is the early reaction of the system to harmful stimuli and later, the chronic inflammation respond based on the conditions of damage to the body. Cyclooxygenase (COX) is the key enzymes in the synthesis of prostaglandins, prostacyclins and thromboxanes which are involved in inflammation, pain and platelet aggregation. Steroidal and non-steroidal anti-inflammatory drugs (SAIDs and NASIDs, respectively) are currently the most widely used drugs in the treatment of acute inflammatory disorders, despite their renal and gastric negative secondary effects. These drugs block COX-1 and COX-2 enzyme activity. COX enzymes assist with prostaglandin production. NSAIDs, steroidal anti-inflammatory drugs are being used till now, As a result long term uses of these drugs cause adverse side effects and damage human biological system such as liver, gastrointestinal tract, etc. (Tamsyn SA Thring et al., 2011).

Based on these studies, the extracts of *S. tenerrimum* is procured & it's anticoagulant, antioxidant, anti-inflammatory activities are tested. The compound that extracted is fucoidan and it has many applications in medicine and human health. Among this property tested in this study anti-inflammatory of fucoidan have not been recorded so far. In previous literature the fucoidan compound has proved to be an effective anticoagulant/antioxidant. The properties of fucoidan as an anti-inflammatory compound have not been tested so far. The potential of fucoidan as an anti- inflammatory drug from *S. tenerrimum* will be explored in this study.

II. MATERIALS AND METHODS

II.1 Collection of seaweed

The brown seaweed *Sargassum tenerrimum* was collected from the Chinnavilai coast, Tamilnadu, (8.1405^{O}N Latitude; 77.3021^{O}E Longitude) India. The collected sample was washed thoroughly with sea water to remove sand particles, pebbles and epiphytes. The samples were then washed with tap water to remove salts and it was finally washed with distilled water. The Samples were then shade dried for 15 days. The dried sample was grounded with blender to get fine powder and it was stored at 4°C for future use.

II. 2 Extraction of fucoidan

The seaweed was washed under running tap water thoroughly to remove sand and impurities. Then it was dried in shade at room temperature for more than 10 days. The dried seaweed was grounded into fine powder and stored for further use. The fucoidan was extracted by the method proposed by Yang et al. (2008). 20g of milled seaweed was treated with 1 liter of ethanol with constant mechanical stirring for 12h at room temperature to remove pigments and proteins. Then washed with acetone and centrifuged at 1800g for 10min. Then the residue was dried at room temperature. From this dried biomass, 5g was taken and extracted with 100ml of distilled water at 65°C with stirring for 1h. The extraction was conducted twice and the extracts were combined. The extract was centrifuged at 18500xg for 10min and the supernatant was collected. The supernatant was mixed with 1% $CaCl_2$ and the solution was kept at 4°C for overnight to precipitate alginic acid. The solution was centrifuged at 18500xg for 10min and the supernatant was collected. Ethanol (99%) was added in to the supernatant to obtain the final ethanol concentration of 30% and the solution was placed at 4°C for 4h. Then the solution was centrifuged at 18500xg for 10min and the supernatant was collected. More ethanol (99%) was added into the supernatant to obtain the final concentration of 70% and the solution was placed at 4°C overnight. The fucoidan was obtained by centrifugation at 18500xg for 10min. Then the fucoidan was dried at room temperature overnight. The

dried fucoidan was packed in an airtight container until further use. The yield of fucoidan was calculated by the following formula. Yield of fucoidan (%) = [Weight of fucoidan/ Weight of milled seaweed] x 100.

II.3 Estimation of total sugars

The fucose content of fucoidan was determined by phenol sulphuric acid method proposed by Dubois et al. (1956). The polysaccharide sample 20 mg was dissolved in 2 ml of distilled water and it served as stock solution. From the stock solution, different volumes (20, 40, 60, 80, 100 µg/ml) was taken in different tubes and made with water up to 100 µl. Then, to that each tube containing the sample 100µl of 89% phenol reagent was added. Then 4.0ml of concentrated sulfuric acid was added to the sample tube and mixed well. Then the solution was kept at room temperature for 10min and the optical density was measured at 490nm by using a spectrophotometer. The distilled water served as blank. For fucose analysis, the commercial L-fucose was used as the standard. The values obtained were plotted in graph and the concentration of fucose was calculated.

II.4 Estimation of protein

Protein content was estimated by Bradford method using bovine serum albumin as the standard (Bradford, 1976). Bovine serum albumin was used as the standard. Various Concentration of standard and Fucoidan Extraction (FE) were added to test tubes and the volume was made up to 1 ml with distilled water. To each tube, 3ml of Bradford reagent (50 ml of Coomassie brilliant blue G250 in 95% ethanol and 100ml of 85% phosphoric acid in 1 liter of distilled water) was added and incubated at room temperature for 10 minutes. The absorbance was read at 595nm. The values obtained were plotted in graph and the concentration of protein was calculated.

II.5 Estimation of Uronic Acid

The glucuronic acid was determined by the method described by Cesaratti *et al.*, 2003. Fucoidan Extraction was dissolved in distilled water at a concentration of 1 mg/ml and 50µl was used for the assay. D-glucuronic acid lactone was used as the standard. Various concentrations of standard and Fucoidan Extraction (50µg) were added to separate wells in a microtitre plate and the volume was made up to 50µl with distilled water. To each well, 200µl of sodium tetraborate (25mM in sulfuric acid) was added and incubated for 10 minutes at 100^{O}C.After cooling the reaction mixture to room temperature for 15 minutes,50µl of carbazole (0.125% in absolute ethanol) was added and incubated for another 10 minutes at 100^{O}C.The absorbance was read at 550nm. The values obtained were plotted in graph and the concentration of uronic acid was calculated.

II.6 Estimation of sulfate content

Sulfate content was determined by benzidine method using sodium sulfate as the standard. Various concentrations of standard and fucoidan extract were dissolved in 25% formic acid at concentration of 1 mg/ml and 1 ml of each was transferred to a fresh tube. To each tube, 1ml of glacial acetic acid, 3ml of acetone/ethanol (1:1) and 1 ml of benzidine (0.5% in 94% ethanol) were added and left overnight at 4^{O}C. The reaction mixture was centrifuged at 5000g for 30 minutes and the pellet was washed in acetone/ethanol(1:1) and suspended in 1.5ml of 1M HCL. The mixture was mixed well and incubated at room temperature for 30 minutes. It was then followed by the addition of 1 ml of distilled water and 0.5ml of 0.1 M sodium nitrite. After 5 minutes, 2.5ml of thymol solution (0.5% in 2M NaOH) was added and the absorbance was read at 505nm. The values obtained were plotted in graph and the concentration of sulfate was calculated.

II.7 Determination of total antioxidant activity

Total antioxidant effect of fucoidan was performed as described by Prieto *et al*. (1999). The fucoidan sample with varying concentrations such as 10, 50, 250, 500 and 1000 µg/ml was mixed with 1 mL of standard reagent solution (0.6 M Sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and were incubated at 95°C. The absorbance was measured at 695 nm against a blank and ascorbic acid served as control. The experiment was repeated three times. % of inhibition = [Control OD – Sample OD/ Control OD] X 100.

II.8 Anti-inflammatory Activity

The anti-inflammatory activity was determined by following Oyedepo *et a1.*, l995). The sample with 100µg/ml was mixed with 40µl BSA and 150 µl PBS and incubated for 20 minutes at 37^{O}C. After

incubation, the mixture was heat inactivated at 57^{O}C for 3 min. Then, the tubes were cooled and optical density was measured at 660 nm. Diclofenac was used as standard. The percentage of inhibition was calculated as % inhibition = [Control OD – Sample OD/ Control OD] x 100.

II.9 Hemolytic activity

In vitro hemolytic activity was performed by spectrophotometer method. A volume of 5 ml blood was collected from a healthy individual and the RBC was collected by centrifugation at 1500 rpm. The pellet was washed three times with sterile phosphate buffer saline solution (pH 7.2±0.2) by centrifugation at 1500 rpm for 5 min. The cells were suspended in normal saline to 0.5%. A volume of 0.5 ml of the cell suspension was mixed with 0.5 ml of the plant extracts (125,250,500 and1000µg/ml concentrations in saline).The mixtures were incubated for 30 min at 37^{O}C and centrifuged at 1500 rpm for 10 min. The free hemoglobin in the supernatants was measured in UV-Vis spectrophotometer at 540 nm. Phosphate buffer saline and distilled water were used as positive and negative controls. The experiment was carried out in triplicates. The level of percentage hemolysis by the sample was calculated according to the following formula, % Hemolysis = ([A_t -A_n]/ [A_t- A_c]) x 100. Where, A_t - absorbance of test sample; A_n- absorbance of saline control; A_c - absorbance of water control.

II.10 Anticoagulant activity

Human blood plasma was collected from healthy donors into conical tubes with 2.5% sodium citrate solution. The plasma was separated from blood cells by centrifuging at 5400 rpm at 4°C for 20 min. The blood plasma was stored at −70°C until use.

II.10.1 Activated partial thromboplastin time (APTT) assay

Anticoagulant activity was determined by activated partial thromboplastin time (APTT) coagulation assay. Briefly, citrated normal human plasma (90L) was mixed with10L of a crude SWE (different concentrations based on the fermentation) of the fermented sample and incubated for 1 min at 37°C. Then, APTT assay reagent (100µL) was added to the mixture and incubated for 5 min at 37°C.Thereafter, 0.05 mM CaCl2 (100µL) was added and clotting time(s) recorded using a Stopwatch. The activity was expressed as relative clotting factor and it was calculated by the formula as follows:Relative clotting factor (R.C.F) = clotting time of test sample/clotting time of control

II.10.2 Prothrombin time (PT)

The PT assay was performed by mixing 90µL citrated normal human plasma with 10 µL of fucoidan and incubated for 10 min at 37°C. After incubation, the PT assay reagent (200 µL) was added to the mixture. The clotting time was recorded with stop clock. Heparin was used as standard.

III. RESULTS

III.1 Chemical Analysis

The yield of crude fucoidan extract from *S. tenerrimum* and their biochemical contents were tabulated (Table1). The results revealed that, the fucoidan extract comprises high sulfate and uronic acid. The total carbohydrate was estimated to be 52.35% w/w. The sulfate and uronic acid contents were found to be 37% and 32.52% respectively. The protein levels were found to be negligible.

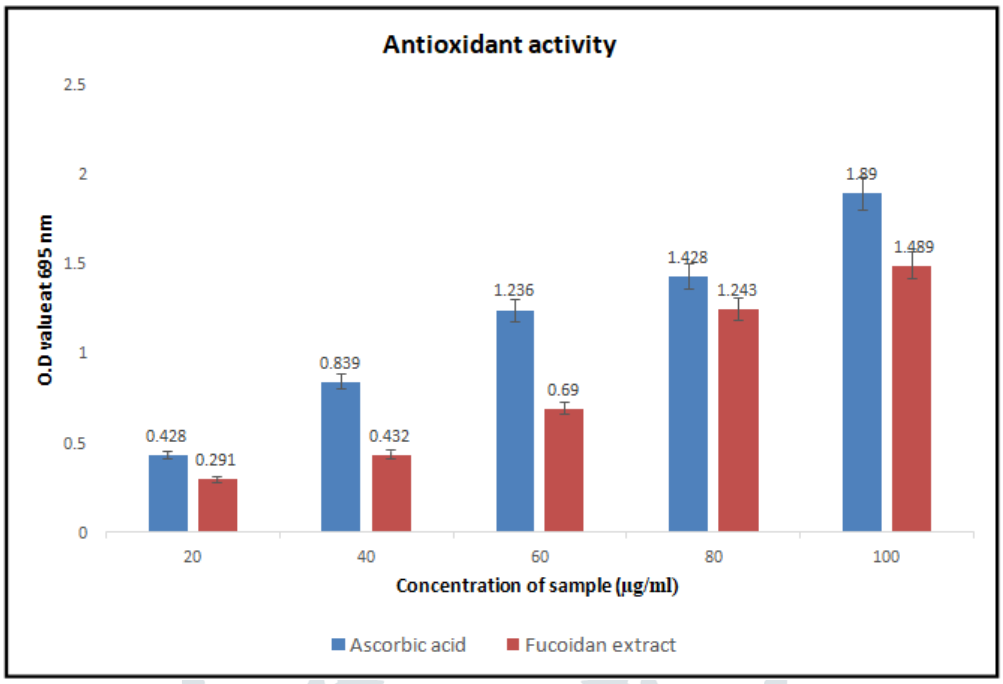
Sample	Yield	Total sugar content (%)	Uronic Acid content (%)	Sulfate content (%)	Protein content (%)
Crude Fucoidan	0.25g	52.35	32.52	37	Very low

*The yield is about 0.5 gm of fucoidan extract from 2% of the dried seaweed powder (25 gm).

Table: 1 Composition and yield of crude fucoidan fractions from S. tenerrimum

III.2 Antioxidant Activity

The total antioxidant activity of fucoidan was depicted in Figure 1. The results showed that the fucoidan activity were found dose dependent and it showed significant inhibition of 91.21% at the maximum concentration 100 µg/ml.



*values are expressed in mean ± standard error

Fig 1: Total antioxidant activity of fucoidan extract from brown seaweed *S. tenerrimum*

III.3. Anti – inflammatory activity and Hemolytic activity:

The fucoidan extract exhibited promising anti-inflammatory activity which has been compared with common drug Diclofenac. The sample with a concentration of 100 µg/ml exhibited 92.60% of activity and the control drug Diclofenac exhibited 97.7% against heat induced protein denaturation which was recorded in table 2. The sample also showed effective activity for haemolysis inhibition was represented in fig 2.

Sample	% of inhibition of protein denaturation
FE (100 µg/ml)	92.60
Control- Diclofenac (100 µg/ml)	97. 7

Table 2: The anti-inflammatory of crude fucoidan extract from *S. tenerrimum* on heat induced protein denaturation

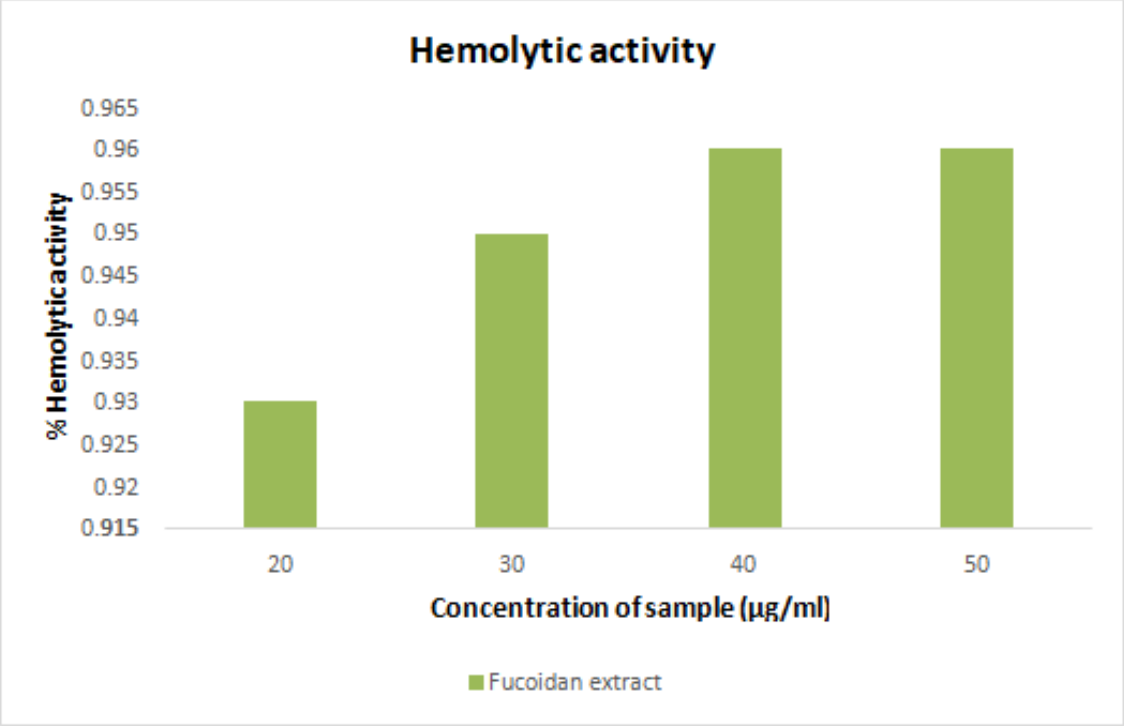


Fig 2: Haemolytic activity of fucoidan

III.4. Anticoagulant activity

Anticoagulant activity of fucoidan extract at five different concentrations was tested in blood plasma was shown in Table 3. Heparin (Positive control) showed the highest clotting time of >1200s at 100 µg/ml. The APTT activity of fucoidan was recorded as >1000 sec at a concentration of 100 µg/ml and minimum of 220 sec at 20 µg/ml whereas, the PT activity of crude fucoidan are maximum recorded to be136 sec at 100 µg/ml. Relative clotting factor was calculated as clotting time of sample (FE)/clotting time of control (water). Heparin (100 µg/ml = >1200). Clotting time >1,000 s considered as 1,000 s to calculate the relative clotting factor

Sample	Concentration (µg/ml)	Clotting time (seconds)			
		APTT	RCF	PT	RCF
Fucoidan	20	220	6.04	35	1.76
	40	415	11.40	63	3.20
	60	618	16.97	89	4.49
	80	834	22.91	119	6.02
	100	>1000	27.47	136	6.86
Water (Control)	30	30	1	12	1

Table 3: Anticoagulant activity of crude fucoidan extract from *S. tenerrimum*

IV. Discussion

Seaweeds have been used as a functional food in many Asian countries; they have been underutilized in countries like India where they grow in abundance. When mixed with diets, seaweed polysaccharides were found to be good for immunity (Teas et al., 1984). In this context, this study was undertaken to identify the biological activities of fucoidan extract from brown algae Sargassum tenerrimum.

The chemical composition of the fucoidan extract from S. tenerrimum was found in accordance with the fucoidan isolated from other brown algae (Souza et al., 2007). However its content varied considerably due to the fact of several factors like environmental condition, seasonal variation, physiological factors and extraction methods (Armis 1995). The fucoidan revealed the total antioxidant activity to be 91.21% at concentration 100µg/ml. In this phosphomolybdenum method, Mo (VI) is reduced to form a green phosphate Mo (V) complex (Nemudzivhadi et al., 2014). As it showed good inhibition rate, this study confirmed the ability of fucoidan in inhibiting the free radicals.

Inflammation is a common manifestation of infectious diseases like leprosy, tuberculosis, syphilis, asthma, inflammatory bowel syndrome, nephritis, vasculitis, celiac diseases, auto- immune diseases etc. (Das et al., 1995). Denaturation of proteins is still one of the major reasons for inflammation process. Therefore, the anti-inflmmatory activity of fucoidan in preventing protein denaturation was attempted. The fucoidan extract exhibited promising anti-inflammatory activity that was compared with common drug Diclofenac. The anti-inflammatory activities of fucoidan extract from S. tenerrimum were reported first time in this study.In the present study, S. tenerrimum fucoidan crude extract showed potential anticoagulant activity. The fucoidan subjected for APTT and PT assay exhibited promising anticoagulant activity, since the fucoidan contains high sulphate content (Melo et al., 2004). Therefore, this present study indicates that hot water extraction of sulfated polysaccharides showed promising activity. As reported previously, hot water was used for the extraction of anticoagulant compounds from marine brown and green algae which was similar to the findings of the present study (Shanmugam and Mody, 2000).

All encompassed, this study explored the potential of fucoidan from S. tenerrimum in relation with antioxidant, anti-inflammatory and anticoagulant activities.

V. Conclusion

Fucoidans are complex sulfated polysaccharides with L-fucose as the main sugar component and are found to possess diverse biomedical potential including anticoagulant, antioxidant, anti-inflammatory etc. The anti-inflammatory activity of fucoidan from *Sargassum tenerrimum* is still not been studied, therefore the present study was carried out. The crude fucoidan extracted from *Sargassum tenerrimum* by hot water extraction and alcohol precipitation was assessed for its biochemical composition and biological potential. The results revealed that, the fucoidan contains high amount of fucose, sulpfate and uronic acid. It also exhibited significant activity in relation with antioxidant, anti–inflammatory and anticoagulant activities. Further in-depth investigations should be carried out on the anti- inflammatory and anticoagulant properties of this fucoidan to understand the molecular mechanisms that might lead the chemical entities for clinical use.

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