Research in Molecular Medicine

Anticoagulative Effect of Two Species of Brown Algae: *Sargassum Angustifolium* and *Cystoseira Indica*

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ABSTRACT

Background: Fucoidans are a group of sulfated fucose-rich polysaccharides that are isolated from brown marine algae and echinoderms, and recently have been found in seagrasses. Fucoidans, as well as their derivatives, have several beneficial biological effects and therapeutic potentials. In the present study, we aimed to evaluate the anticoagulative effects of two species of brown algae, namely *Sargassum angustifolium* (*S. angustifolium*) and *Cystoseira indica* (*C. indica*).

Materials and Methods: Fucoidan C and fucoidan S were extracted by an ethanol/water solvent system from S. angustifolium and C. indicia, respectively. The anticoagulative effects of fucoidan C and fucoidan S were tested on 10 normal serum samples by evaluating the rate of thrombin time (PT) and Prothrombin Time (PTT).

Results: Both fucoidan C and fucoidan S significantly increased PTT. However, no significant difference was observed in PT. Fucoidan C had a greater effect on PTT prolongation compared with fucoidan S.

Conclusion: Both fucoidans extracted from S. angustifolium and C. indicia can be used as anticoagulants in biotechnology and human disorders.

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Introduction

Like other herbs, algae contain various organic and inorganic materials that can be used for therapeutic purposes. Seaweed is a rich source of bioactive compounds that can produce a variety of metabolites with a wide range of biological activities. Many microalgae and macroalgae are suitable sources of carbohydrates with many applications due to their bio-structural properties. Marine polysaccharides (mainly chitin, chitosan, fucoidan, carrageenan, and alginate) affect proliferation, cell cycle, and the regulation of various metabolic pathways, such as antioxidant, antibacterial, anti-viral, immune-stimulating, anticoagulant, and anti-cancer [1].

In 1913, scientists found that brown algae had anticoagulant properties [2]. These polysaccharides have beneficial effects on human health and used in cosmetics and food products [1]. In the cell wall of brown algae, fucoidans, which are complexes of sulfated polysaccharides, are widely found [1]. Fucoidans have therapeutic properties that increase their healing potential with their degree of sulfation. Among the fucoidan products, isoproterenol increases serum levels of creatine phosphokinase, lactate dehydrogenase, and transaminase in animals, which are involved in myocardial infarction. Fucoidan has been shown to possess therapeutic properties by reducing serum levels of these enzyme markers for the treatment and prevention of heart attacks. Besides, fucoidan reduces the activity of lipid peroxidation and increases the antioxidant enzyme and, therefore, decreases myocardial infarction risk [3]. Free radicals prevent the release of enzymes such as catalase, superoxide dismutase and glutathione peroxidase, which are the first line defense against oxidative damage. The equilibrium between these enzymes is critical by effectively eliminating the oxygen species in intracellular organelles.

The second line of defense consists of non-enzyme inhibition by α-tocopherol and ascorbic acid, an inhibitor of free radicals, by antioxidant enzymes [4]. The vascular damage caused by plaque rupture, endothelial structural abnormalities, and endothelial cell analysis in the presence of prothrombin significantly increase the risk of thrombosis.

Myocardial infarction, which occurs in acute coronary syndrome, is often associated with coronary artery thrombosis, leading to complete or incomplete luminal blockage [5]. Each coagulation factor (proenzyme), except fibrinogen, is activated step-by-step to thrombin but, fibrinogen becomes fibrin. It has been reported that heparin, as an anticoagulant, can inhibit the activity of coagulation factors, including thrombin, XIIa, XIa, Xa, and IXa with antithrombin III [6-10]. In inflammatory diseases, the enzyme activation leads to physiological, biochemical, and pathological changes [2]. With the rapid advances in medical research, many therapeutic methods have been developed for severe inflammatory diseases, but they often have adverse side effects [11, 12]. Several anticoagulants have been introduced, but heparin is still suggested as an anticoagulant for the treatment of acute thrombosis. Although the clinical effects of heparin are significant, it has side effects. Bleeding occurs in 1% to 33% of patients receiving heparin. Another critical side effect of heparin therapy is heparin-induced thrombocytopenia, eosinophilia, skin reactions, allergic reactions thrombocytopenia, alopecia, elevated liver function tests, and increased potassium level [13, 14]. Therefore, heparin therapy is highly problematic [15]. Anticoagulant and antithrombotic properties of 250 marine species of brown algae have been reported [11], most of which belong to class Phaeophyceae [13, 14].

Over the past decade, numerous studies have been conducted on biological activities of agents derived from marine species, including anticoagulation, antithrombosis, immunomodulation, and anti-inflammatory features [16]. Also, extensive studies have investigated its anticoagulant potential [17, 18]. The structural study of fucoidan species from Ecklonia kurome has reported the anticoagulant properties of this species due to its sulfated fucans [19, 20]. Studies show that the brown algae crude fucoidan also has anticoagulant effects [19]. Studies of 9 species of brown algae have shown that polysaccharide sulfate from fucoidan regulates the process of coagulation [21]. In the present study, the coagulation effects of two species of brown algae, namely S. angustifolium and C. indicia were investigated.

Materials and Methods

Study population

A total of ten serum samples (five males and five females) were taken from healthy subjects with the inclusion criteria. The Mean±SD age of the subjects was 32.60±9.77 years. To evaluate the performance of fucoidans in different conditions in terms of coagulation factors, individuals with different values of PT and PTT entered the study. The inclusion criteria of the study population were the absence of any cardiovascular disease, hypertension, diabetes, kidney diseases, and hemophilia A or B. Subjects had no recent usage of aspirin or other Non-Steroidal Anti-Inflammatory Drugs (NSAID) and did not take any food within the last eight hours before sampling.

Extraction of fucoidans

S. angustifolium and C. indica were obtained from the coast of Bushehr, Iran, and were transferred to the Persian Gulf Institute for identification. To prepare the extract, the collected algae were washed immediately with seawater, and the mud and other sticking substances were removed. The samples were dried in shade for four days. Subsequently, the samples were placed in zipper plastic bags. For extraction, 20 g of algae with 400 mL of distilled water was placed on a stirrer at 45°C for 45 minutes. At pH 5 and 50°C, 1 g of sodium chloride (NaCl) was added that reached pH to 7. Then, by adding 0.1 M NaOH, pH reached 7.59 and the mixture was maintained at 45°C for 3 h at pH=7 (the mix was still on the stirrer). After an hour, 24 g NaCl (1%) was added. The resulting compounds were precipitated by adding 100 mL of absolute ethanol. The final solution was kept for one night at room temperature. On the second day, the mixture was centrifuged and the supernatant was removed. Again, the compounds were precipitated with 500 mL of water twice a day at room temperature for 30 to 60 min on a stirrer. Then, 9 g of NaCl was dissolved in water and distilled twice and then added to the incubating samples. Afterward, 100 mL of absolute ethanol was added to the mixture and maintained for one night at room temperature. On the third day, the mixture was passed through a filter, the remaining mixture on the filter was dissolved with 150 mL twice-distilled water, and was placed on a stirrer for 1 h at 40°C. Using a 2 N HCl solution, the pH adjusted to 3 and filtered using a 0.2-µm filter. The resulting solution was transferred to the freeze-dryer device. The composition was finally prepared in powder form after 24 hours.

Biochemical analysis of fucoidans

Monosaccharide analytical chemistry and structure characterization of fucoidans were performed by High-Performance Liquid Chromatography (HPLC) and Fourier-Transform Infrared Spectroscopy (FTIR) spectrophotometry, respectively. The hydrolyzed polysaccharide sample (90 min in 2 M trifluoroacetic acid at 120°C) is injected into the HPLC system (VARIAN, Pro Star, USA). An acetonitrile/deionized water mixture (90:10) was used as the mobile phase at a flow rate of 2 mL/min. Then, FTIR analysis (PerkinElmer FT-IR, Spectrum RXI, USA) was performed on the grinded sample in potassium bromide (KBr). Signals were collected automatically using 60 scans ranging 400-4000 cm−1 at a resolution of 32 cm−1.

Preparing fucoidan solutions

One gram of powder was dissolved in 50 mL of distilled water and was placed on a shaker at 25°C for 12 hours. The resulting solution (0.02 g/mL) was filtered (0.4 µm) and used to check for the coagulative effects on 10 serum samples from healthy individuals.

PT assay

First, 200 µL of PT solution (Fisher Scientific, USA) was put into a water bath at 37°C. Then, 100 µL of the fresh plasma was added into glass tubes containing 200 µL of PT solution, 190 µL of PT solution plus 10 µL of normal saline, and 190 µL of PT solution plus 10 µL of fucoidan C or S. Immediately afterward, the stopwatch was set. While the tube was shaking, it was under precise observation until the formation of fibrin fibers was observed. After observing the first fibrin, we recorded the time and reported it as the PT value.

PTT assay

To warm the PTT solution (Fisher Scientific, USA) to 37°C, they were placed in a water bath. Then, 100 µL of PTT solution, 90 µL of PTT solution plus 10 µL of normal saline, and 90 µL of PTT solution plus 10 µL of fucoidan C or S were added to glass tubes inside the water bath. Then 100 µL of the fresh plasma was added into each tube. After 2 minutes, 100 µL of CaCl2 (37°C), which was already placed in a glass tube inside the water bath, was added into each tube. As soon as CaCl2 was added, the stopwatch was turned on and the tube was shaken to form fibrin and was reported as PPT value.

Statistical methods

SPSS V. 25 (SPSS, Chicago, IL, USA) was used to analyze the obtained data. The Kolmogorov-Smirnov test was used to measure the normality of data distribution. The Kruskal-Wallis one-way analysis of variance was used to compare groups. GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA, www.graphpad.com) was used to plot the graphs. The obtained data were expressed as Mean±SD. $P<0.05$ was considered to be statistically significant.

Results

Biochemical analysis of fucoidans

Monosaccharide composition analysis showed that fucose was the main sugar in the chemical composition (63%±2.0%) and the lowest amount was related to glu-

Table 1. Effect of fucoidan *Cystoseira indica* on PT and PTT

Test Group	Mean±SD Time (s)	P
PT	14.55±4.01	
PT with Normal Saline	15.55±4.01	0.31
PT with Fucoidan C	14.90±3.66	
PTT	35.10±4.99	
PTT with Normal Saline	38.10±4.99	0.0002
PTT with Fucoidan C	181.40±64.16	

PT: Thrombin Time; PTT: Prothrombin Time

Table 2. Effect of fucoidan *Sargassum angustifolium* on PT and PTT

Test Group	Mean±SD Time (s)	P
PT	12.96±1.71	
PT with Normal Saline	13.96±1.71	0.12
PT with Fucoidan S	13.85±1.49	
PTT	32.30±6.21	
PTT with Normal Saline	37.30±6.21	0.004
PTT with Fucoidan S	83.00±38.81	

PT: Thrombin Time; PTT: Prothrombin Time

cose (6%±0.4%). Furthermore, the FTIR results showed that the peaks obtained in the different spectra of absorption bands corresponded to the standard fucoidans absorption spectrum.

Effect of fucoidan C on PT and PTT

It was observed that fucoidan C caused a slight difference in PT when compared with normal saline and control group and the difference was not significant (P=0.31; Table 1, Figure 1A). However, fucoidan C (181.40±64.167 s) culminated in a significant increase in PTT when compared to normal saline (38.10±4.99 s) and control group (35.10±4.99 s; P=0.0002; Table 1, Figure 1B)

Effect of fucoidan S on the PT and PTT

Our experiments demonstrated that fucoidan S caused no statistically significant difference between the three groups (P=0.12; Table 2, Figure 2A). Nonetheless, fucoidan S (83.00±38.81 sec) caused a statistically significant increase in PTT when compared with the normal saline (37.30±6.21 sec) and control group (32.30±6.21 sec; P=0.0002; Table 1, Figure 1B).

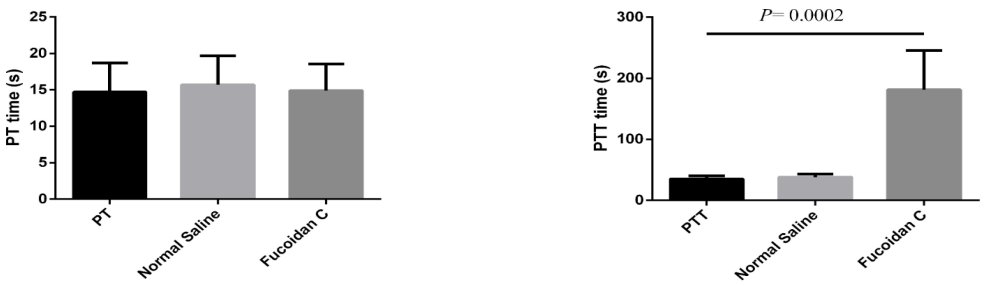

Figure 1. Bar graphs to illustrate the anticoagulative effect of fucoidan C
The Kruskal-Wallis test was used to compare the groups.

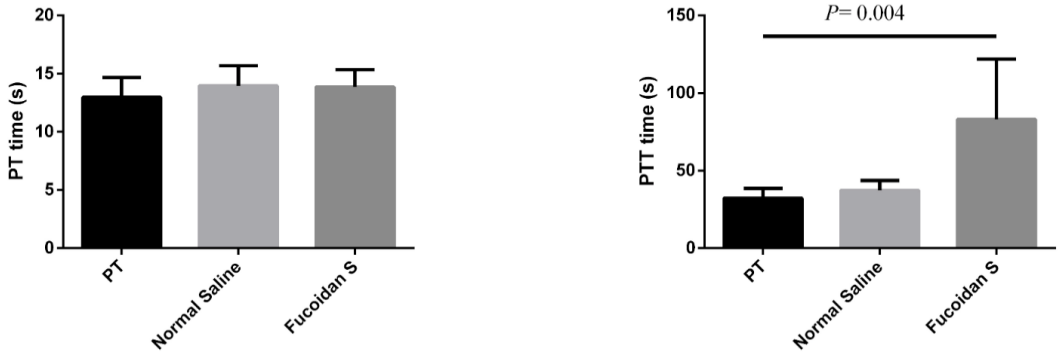

Figure 2. Bar graphs to illustrate the anticoagulative effect of fucoidan S
The Kruskal-Wallis test was used to compare the groups.

Comparison of the effect of fucoidan C and fucoidan S on PTT

Comparing the effect of fucoidan C and fucoidan S on PTT indicated that fucoidan C prolonged PTT further than fucoidan S (P=0.0036, Figure 3).

Discussion

The current study was mainly evaluated the anticoagulative effects of two species of brown algae, S. angustifolium, and C. indica extracts on the serum samples. Our experiments indicated the positive effects of both extracts on increasing the PTT rate. With respect to little adverse effects of natural products, it seems that S. angustifolium and C. indica can be used in patients with an increased coagulative capacity of the blood.

Fucoidan function is similar to that of the mammalian heparin. Heparin is an extensively utilized biological agent with antithrombotic activity; however, the desired antithrombotic effect is very complicated to achieve without increasing the risk of hemorrhage, as heparin also has pronounced anticoagulant effects. A study on mice indicated that Undaria pinnatifida fucoidan achieved an antithrombotic effect without any increase in clotting time, and could be developed for this purpose [22]. Conversely, it was observed that Fucus vesiculosus fucoidan could prolong clotting time, which once again showed differences in the effect of fucoidans.

Moreover, with new research on the pro- and anti-coagulant effects of components of Fucus vesiculosus, research on the use of fucoidans as a pro-coagulant factor in hemophilia cases is progressing [23]. This investigation showed that a minimum size of fucoidan polysaccharides is required to exert an effect on coagulation. They found that fucoidan requires a minimal charge density of 0.5 sulfates per sugar unit and a size of 70 sugar units to demonstrate desired pro-coagulant activities for improvement of hemostasis in factor VIII/factor IX-deficient plasma.

Despite the promising results in investigating the potential scopes of usage, fucoidan has not yet been completely promoted to the level of a routine therapeutic agent. The reasons for such an issue may originate from several concerns. Fucoidan is regarded as a generic term for a class of moieties, and studies cover a wide spectrum of algal and echinoderm fucoidan sources above and beyond various extraction approaches. To attain a

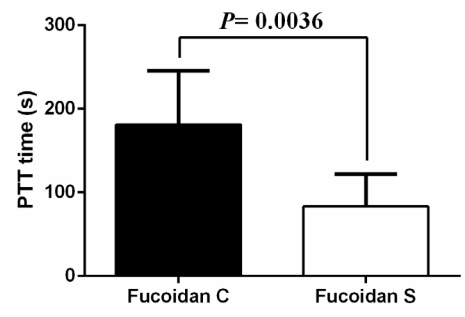

Figure 3. Comparison of the effects of fucoidan C and fucoidan S on PTT

regulated product, fucoidan should be characterized and its reproducibility should be approached. Regular, clean, and programmed harvesting of a single type of seaweed is needed to attain a more reliable product. Coastal areas might be a suitable alternative for harvesting fucoidans that originate from expensive and low-yielding sources like sea cucumbers [24]. Companies must register the product as a patent since there is an interest to transform a product from a preclinical level to clinical trials and ensure their applicability. Several recent patents establish the utilization of fucoidan through the biomaterial scaffolds as well as to treat clotting impairments [25, 26].

To achieve the therapeutic regulatory requirements, it is required to characterize the pharmacokinetics, bioavailability, and distribution of specific fucoidans. Currently, the approaches and tools to ensure such measurements are becoming available. On the other hand, the safety of fucoidans has been established and has been available as food or dietary supplements [27, 28]. As a result, fucoidans are probably used as supplementary products currently in the short term. Nonetheless, further research might introduce them as promising products to be used in clinics. Moreover, the fractions of fucoidan extracts should be precisely characterized to attain the most beneficial results.

Altogether, we tested the anticoagulative properties of fucoidan S and fucoidan C extracted from two species of brown algae, S. angustifolium and C. indica, respectively, on the serum samples of healthy individuals. Experiments testified that both extracts increased the PPT rate, but fucoidan C was much more efficacious in prolonging the PTT that fucoidan S. With respect to a few adverse effects of natural products, it seems that S. angustifolium and C. indica can be used in patients with an impaired coagulative capacity of blood as well as those with a high risk of heart diseases due to increased clotting rate.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article.

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Authors' contributions

Conceptualization, supervision: Mohammad Javad Mousavi, Narges Obeidi; methodology: Narges Obeidi; Investigation and writing – original draft: Arghavan Hosseinpouri, Hamideh Malekhayati, Elham Ehsandoost; Review & editing: Mohammad Javad Mousavi; Funding acquisition, Resources: Narges Obeidi.

Conflict of interest

The authors declared no conflict of interest.

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