Effect of ethanolic extract from brown seaweed *Sargassum wightii* and its polysaccharide; fucoidan on inhibition of α-amylase and α-glucosidase activities - An *in vitro* study

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Running title: Effect of *S. wightii* on diabetes indicator enzymes

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Abstract

Introduction: One of the modes of diabetes treatment is the inhibition of diabetes inhibitor enzymes like α-amylase and α-glucosidase. The search for natural drugs with fewer side effects has led to the identification of certain species of seaweeds showing inhibition of these enzymes. This study deals with the effectiveness of ethanolic extract of the brown seaweed *Sargassum wightii* and its polysaccharide; fucoidan on α-amylase and α-glucosidase using *in vitro* study. **Materials and methods:** Crude extract and fucoidan were prepared from *S.Wightii.* The standard drug used for comparison was acarbose. The inhibitory effect of the seaweed derivatives on α-amylase and α-glucosidase were analysed. **Results:** IC_{50} values for α-amylase inhibitory activity were 394.2 µg/mL for S.wightii ethanolic extract, 222.6 µg/mL for fucoidan, and 96.6 µg/mL for acarbose. Statistical analysis by two-way ANOVA indicated that the variation in inhibitory activity due to concentration was statistically significant ($P < 0.001$) for all the tested samples including acarbose. Regarding the α-glucosidase enzyme, irrespective of the tested samples, a concentration-dependent inhibitory activity was noted. The IC_{50} values for α-glucosidase inhibitory activity were 320.7 µg/mL for *S.wightii* ethanolic extract, 196.0 µg/mL for fucoidan, and 42.2 µg/mL for acarbose, respectively. Two-way

ANOVA indicated that the variation in α-glucosidase activity due to the concentration of tested samples and acarbose was statistically significant ($P < 0.001$). **Conclusion:** The inhibitory effect on α-amylase and α-glucosidase by the isolated molecule; fucoidan from S. wightii was higher compared to the crude ethanolic extract. Hence, fucoidan from the seaweed *S. wightii* can be used as an adjunct for the treatment of diabetes.

Key words α-amylase, α-glucosidase, Sargassum wightii, seaweed, fucoidan

Introduction

The most consumed nutrient in the human diet is carbohydrates, of which amylopectin constitutes the major part. After food intake and during metabolic processes, α-amylase and α-glucosidase enzymes accelerate the hydrolysis of starch and aid the release of glucose into the bloodstream. Moreover, routine consumption of carbohydrate-rich diets, unhealthy lifestyles, and genetic disorders lead to abnormal blood glucose levels after a meal, known as postprandial hyperglycemia. This has been associated with the development of oxidative stress in cells, hypertension, elevated cholesterol, obesity, and metabolic syndrome (1-3). This condition ultimately leads to the development of diabetes mellitus Type 2, cardiovascular disease, and cancer (3). Worldwide, these diseases cause death and pose great economic loss in the public health system, especially in developing countries. To overcome these chronic diseases, efforts have been made to prevent or lower the risk factors associated with postprandial hyperglycemia. With this concern, the administration of acarbose is considered a viable therapy, which lowers glucose absorption and decreases postprandial hyperglycemia by inhibiting the activity of α-amylase and α-glucosidase enzymes. Despite its control over the foresaid enzymes, acarbose causes adverse gastrointestinal side effects due to excessive inhibition of α-amylase (3). To overcome this barrier, scientists all over the world focussed their attention to identify alternative drugs with more efficacy, safety, and fewer side effects.

In this scenario, the use of natural products with active, bioactive molecules provides an alternative source to combat the pathogenesis associated with hyperglycemia (4). Marine seaweed (algae) are non-flowering, photosynthetic organisms, that have been used in traditional medicine as an immune booster, mineral supplement, anticancerous, antiviral, antidiabetic, and also to treat obesity since ancient times. In certain parts of the world, seaweed products are being consumed by humans due to their safety. Also, the marine environment is considered as a potential reservoir which can be judiciously explored to obtain life-saving compounds. In recent years, several compounds have been isolated from marine algae and are reported to possess varied biological activities. Pharmacological activities such as antioxidant, antibacterial, antiviral, cardioprotective, anti-inflammatory, antiulcer, antihyperglycemic, and wound healing are due to their major phytochemical constituents such as flavonoids, alkaloids, saponins, tannins, and polysaccharides (5,6).

Seaweeds inhabit harsh environments and are exposed to varied biotic and abiotic stress factors such as respiratory, desiccation, fluctuating salinity, temperature, and irradiation (7,8). Consequently, their biochemical composition varies according to species and season collection. These variations in turn reflect on the quantity, quality, and efficiency of the biomolecules. Earlier studies during the last decades were made mainly on screening the efficiency of biologically active compounds from seaweeds against various human pathogenic infections, hyperglycemic conditions, and their associated disorders. Generally, the crude

extract is focused more on the screening the biological efficiency of seaweeds, than the specific phytochemicals. Hence, the present study aimed at the screening the inhibitory efficiency of ethanolic extract, and seaweed polysaccharide; fucoidan on the carbohydrate metabolic enzymes such as α-amylase and α-glucosidase as a measure of combating the hyperglycemic disorder.

Materials and Methods

Collection of seaweeds

The seaweed *Sargassum wightii* was collected from the intertidal coastal water of the Gulf of Mannar (Latitude 8° 55'- 9°15 N and Longitude 78°C- 79°C 16E), Tamilnadu during low tide with the help of local fishermen by handpicking. It was identified at Manonmaniam Sundaranar University (Tamilnadu, India)). The collected seaweed was washed immediately and rinsed in the filtered seawater to remove the sand particles and associated epiphytic organisms. Then it was transported to the laboratory in a clean container at a low temperature and rewashed in fresh water to remove the adherent particles and salt. The seaweed was then dried in shade and powdered in a domestic dry mixture. The powdered algal material was stored in a container and was used for the preparation of ethanolic extract, and isolation of the polysaccharide; fucoidan.

Extraction of seaweed extract

Dried seaweed powder of 20.0 g was added in Soxhlet apparatus having 200 mL of 80% ethanol. The extraction was performed for 72 hours and the extract was collected, filtered by using Whatman No.1 filter paper. The obtained extract was concentrated and dried at room temperature to obtain crude extract powder. The crude seaweed extract powder was stored in a labeled container and stored at 20^{o}C until further use. All chemicals used in this investigation were of analytical grade.

Extraction and Purification of fucoidan

In this study, fucoidan from brown seaweed *S. wightii* was extracted and purified by following the method described by Yang et al and Immanuel et al (9,10). The dried brown seaweed, *S.wightii* powder weighing 20.0 g was treated with 1L of 85% ethanol with constant stirring and incubated for 12h at room temperature to remove pigments and proteins. This mixture was washed with acetone and centrifuged at 1800× g for 10 min. The supernatant was discarded and the residue was dried at room temperature. From this dried biomass, 5.0 g was dissolved in 100 mL of distilled water, boiled at 65 °C for 1h with stirring and the extract was collected. This was done twice and all the collected extracts were pooled. This combined extract was centrifuged at 18500× g for 10 minutes and the supernatant was collected. To this supernatant, 99% ethanol was added till 30% concentration is reached and was incubated at 4 °C for 4h. Then this solution was centrifuged at 18500× g for 10 minutes and the supernatant was collected. Again 90% ethanol was added to the supernatant to get a final concentration of 70% ethanol and reincubated overnight at 4°C. Then the solution was filtered through a 0.45 µm pore size nylon membrane and the filtrate was washed with 99% ethanol and acetone, dried at room temperature. The fucoidan thus obtained was stored in an airtight container at room temperature.

The yield of fucoidan obtained was 2.8 ± 0.2%. Further, the fucose content was analysed by phenol sulphuric acid method and the sulfate content was analysed by barium chloride

method (11). The fucose and sulfate contents of fucoidan showed were; 70.5 ± 2.1% and 45.0 ± 1.20 respectively.

***In vitro* analysis of inhibition of α-amylase and α-glucosidase enzymes**

In vitro analysis of α-amylase and α-glucosidase were carried out using the standard methods followed by Apostolidis et al (12).

α-amylase inhibition assay:

A mixture of 500 µL extract or acarbose and 500 µL 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (13U/mL) were incubated at 25 ◦C for 10 min. After preincubation, 500 µL 1% soluble starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) were added to each tube at timed intervals. The reaction mixtures were then incubated at 25 ◦C for 10 min followed by addition of 1 mL dinitrosalicylic acid color reagent. The test tubes were then placed in a boiling water bath for 5 min to stop the reaction and cooled to room temperature. The reaction mixture was then diluted with 10 mL distilled water and absorbance was read at 540 nm. α-amylase inhibitory activity was expressed as inhibition percent and was calculated using the following formula.

$$\%\ \text{inhibition} = (\Delta\text{Abscontrol} - \Delta\text{Abssample} \div \Delta\text{Abscontrol}) \times 100$$

The inhibitory activity was expressed as the half maximal inhibitory concentration (IC50), which is a measure of the effectiveness of a compound in inhibiting biological or biochemical function.

α-glucosidase inhibition assay:

A mixture of 50 µL extract or acarbose solution and 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/mL) was incubated in 96 well plates at 25 ◦C for 10 min. After preincubation, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) were added to each well at timed intervals. The reaction mixtures were incubated at 25 ◦C for 5 min. Before and after incubation, absorbance was recorded at 405 nm by micro-plate reader and compared to that of the control which had 50 µL buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as inhibition percent and was calculated as follows using the following formula.

$$\%\ \text{inhibition} = (\Delta\text{Abscontrol} - \Delta\text{Abssample} \div \Delta\text{Abscontrol}) \times 100$$

The inhibitory results were expressed as the half maximal inhibitory concentration (IC50), which is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. All the chemicals used were of analytical grade and the experiments were conducted six times.

Statistical analysis

The results obtained in the present study were subjected to statistical analysis such as mean and standard deviation. The mean was analyzed by two-way analysis of variance (ANOVA). Statistical analysis and plotting of graphs were carried out using SigmaPlot 13.0 (Systat Software, USA). P-value < 0.05 is considered as statistically significant.

Results

***In vitro* α-amylase inhibitory activity**

The results on the *in vitro* α-amylase activity by the tested seaweed products and standard acarbose are depicted in Figure 1. Ethanolic extract of seaweed *S. wightii* showed potent α-amylase inhibitory activity and it was found to be concentration-dependent. At the lowest

concentration of 50 µg/ml, *S. wightii* ethanolic extract showed 19.8 ± 1.6% inhibitory activity. At the same concentration, fucoidan showed the percentage inhibition of 30.4 ± 1.7% as against the highest inhibition of 64.1 ± 0.8% by the acarbose ($P < 0.0001$). At the highest concentration of 500 µg/ml, the inhibitory activities noted for ethanolic extract, fucoidan, and acarbose were; 59.4 ± 1.1%, 83.4 ± 1.1%, and 99.0 ± 0.1%, respectively. In the other concentrations of the test samples (100 to 450 µg/mL), the range of inhibitory activity noted were; from 25.4 ± 1.40% to 54.2 ± 1.5% in ethanolic extract, from 34.8 ± 1.3% to 76.2 ± 1.4% in fucoidan, and from 70.9 ± 1.1% to 98.5 ± 0.1% in acarbose. Further, concerning the variation in inhibitory efficiency, the IC_{50} values were also varied much and it was 394.2 µg/mL for ethanolic extract, 222.6 µg/mL for fucoidan, and 96.6 µg/mL for acarbose. Statistical analysis by two-way ANOVA indicated that the variation in inhibitory activity due to concentration was statistically significant ($P < 0.0001$) for all the test samples including acarbose.

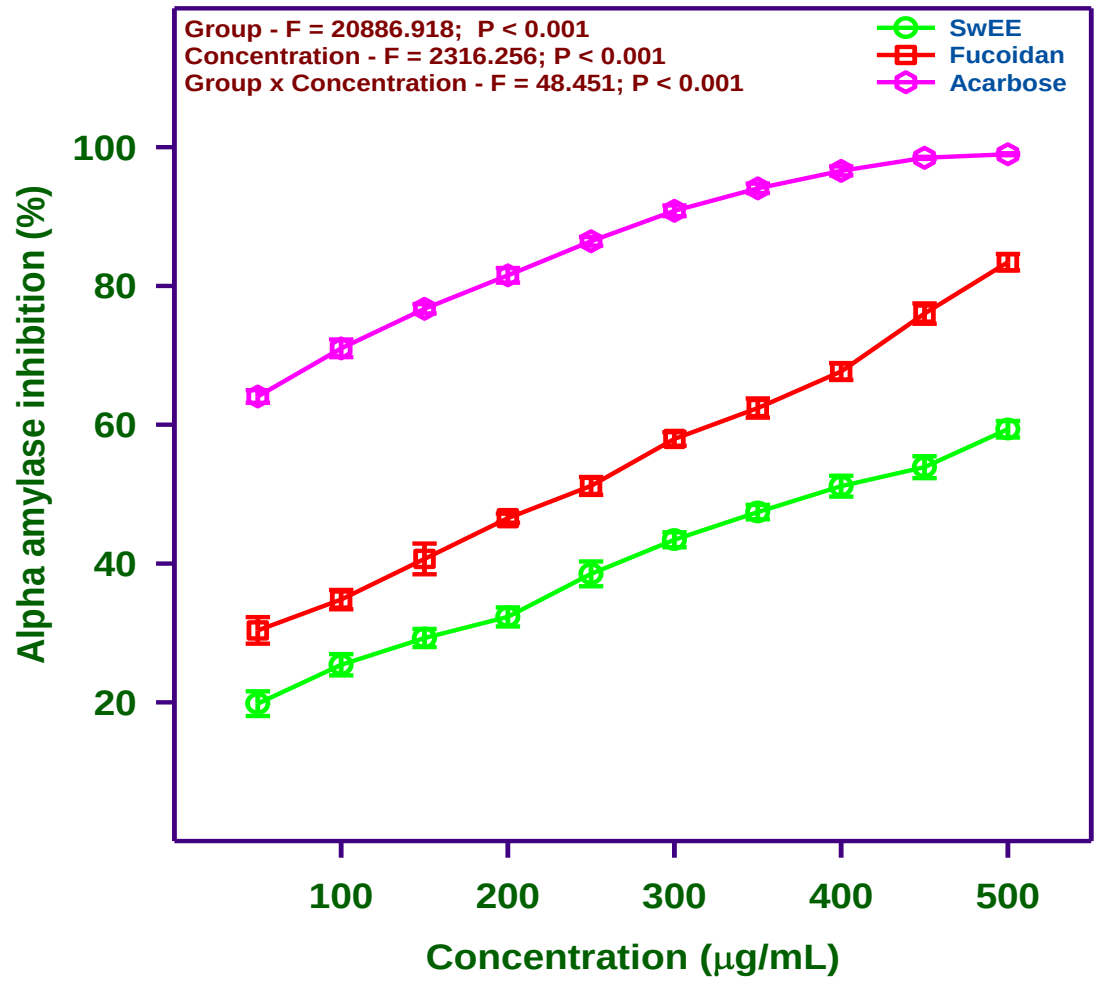

Figure 1: *In vitro* α-amylase inhibitory activity of *S.wightii* ethanolic extract (SwEE), fucoidan, and acarbose. Values are mean ± SD (n=6)
The 'F' and 'P' Values are by two-way ANOVA.
IC50 values are 394.2 µg/mL, 222.6 µg/mL, and 96.6 µg/mL for *S.wightii* ethanolic extract (SwEE), fucoidan, and acarbose respectively.

***In vitro* α-glucosidase inhibitory activity**

The results of the *in vitro* α-glucosidase activity by the tested seaweed products and standard acarbose are depicted in Figure 2. Irrespective of the test samples, the α-glucosidase inhibitory activity was found to be concentration-dependent. At the lowest concentration of 50 µg/mL, the minimum inhibitory activities of 24.0 ± 0.9%, 33.7 ± 1.1%, and 67.8 ± 0.8% were recorded respectively for seaweed ethanolic extract, fucoidan, and acarbose. However, at the highest concentration of 500 µg/mL, the maximum inhibitory activity of 68.0 ± 0.9% for seaweed ethanolic extract, 83.8 ± 1.1% for fucoidan, and 97.7 ± 0.6% for acarbose were noted. In the other tested concentrations (100 to 450 µg/mL), the percentage of inhibitory activities noted were ranged in between the minimum and maximum values recorded for the respective experimental seaweed products inclusive of acarbose. Furthermore, corresponding to the difference in inhibitory activity, the IC_{50} values also exhibited variation among the tested seaweed products and it was 320.7 µg/mL for ethanolic extract, 196.0 µg/mL for fucoidan, and 42.2 µg/mL for acarbose, respectively. The two-way ANOVA tested indicated that the variation in α-glucosidase activity due to concentration of tested seaweed samples and also acarbose was statistically significant ($P < 0.0001$).

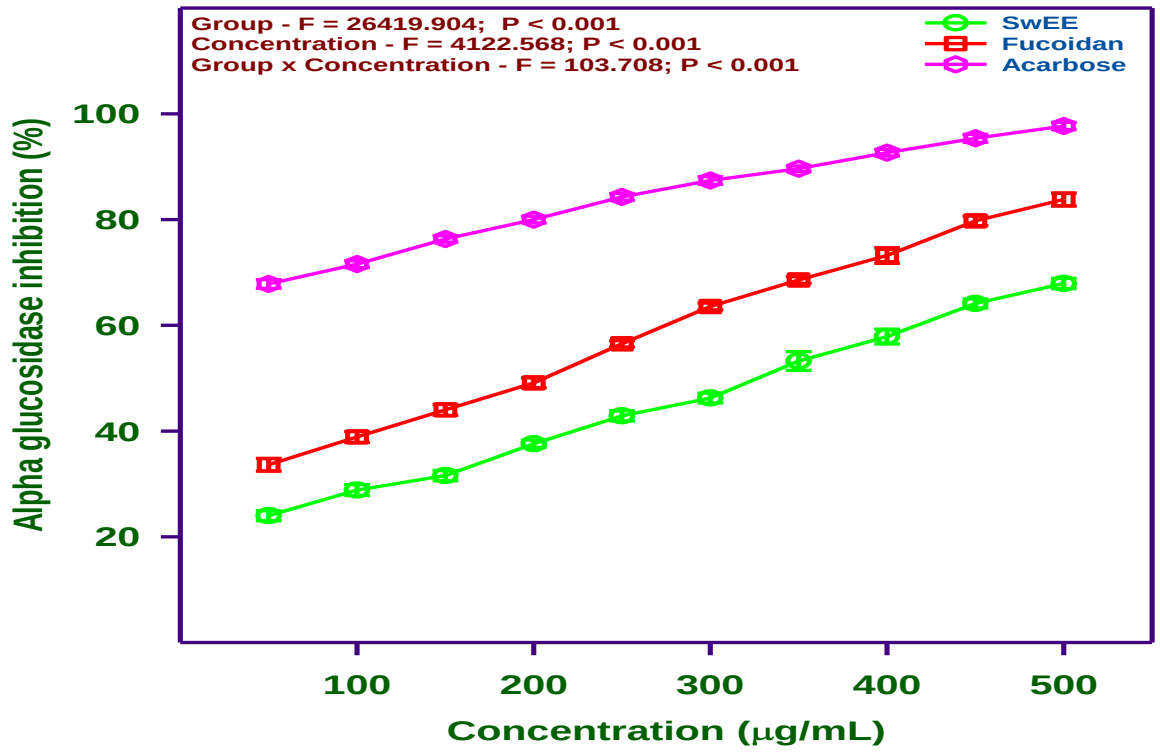

Figure 2: *In vitro* α-glucosidase inhibitory activity of *S.wightii* ethanolic extract (SwEE), fucoidan, and acarbose. Values are mean ± SD (n=6)
The 'F' and 'P' Values are by two-way ANOVA.
IC_{50} values are 320.7 µg/mL, 196.0 µg/mL, and 42.2 µg/mL for *S.wightii* ethanolic extract (SwEE), fucoidan, and acarbose respectively.

Discussion

Inhibition of α-amylase and α-glucosidase enzymes delays the digestion of starch, preventing the abrupt increase in postprandial blood glucose levels (13, 14). This is a key factor in the prevention of obesity and carbohydrate metabolic disorders (2, 3). Acarbose is a pseudo tetrasaccharide isolated from bacteria *Actinoplanes* sp., which acts as a competitive and reversible inhibitor of both α-amylase and α-glucosidase enzymes to reduce postprandial hyperglycemia. This standard drug, attaches to the carbohydrate-binding site of the enzymes with an affinity exceeding that of the normal substrate due to the presence of the intramolecular nitrogen, arresting the enzymatic reaction because of the uncleaved nature of the C–N bonds in the acarviosin unit of acarbose (1,15). However, only mild α-amylase inhibition is recommended, due to the abnormal fermentation of indigestible carbohydrates, which leads to gastrointestinal side effects (13).

To overcome this defect, non-toxic and more selective inhibitors from natural products are used to prevent postprandial hyperglycemia and the associated risks (16, 17). Seaweeds and their phytochemicals have been identified as valuable sources of nutraceuticals (18). In this context, the present study was performed to assess the efficiency of ethanolic extract, and its polysaccharide; fucoidan α-amylase, and α-glucosidase inhibition.

The present results suggest that among the tested seaweed products, the isolated molecule; fucoidan displayed maximum enzyme inhibition activity compared with the ethanolic extract. Specifically, the α-amylase inhibition was high in 50 to 500 µgm/mL fucoidan-added medium when compared with the ethanolic extract. A similar reading was noticed for the α-amylase activity added with 50 to 500 µg/mL acarbose. The variation in α-amylase inhibition in varying concentrations was statistically significant ($P < 0.001$) for the tested seaweed products and the standard drug. In congruence with the changes in α-amylase, the IC_{50} values also showed the variation, and it was 40.9% low for fucoidan compared with the ethanolic extract. Further, in the present study, maximum α-glucosidase inhibition was noted in the fucoidan-added medium against the minimum inhibition noted in the ethanolic extract-added medium. In the standard drug, acarbose-added medium, the α-glucosidase inhibition was higher than the values noted by fucoidan. In all the tested products including acarbose, the variation in the α-glucosidase activity in varying concentrations was statistically significant ($P < 0.001$). Following the changes in the inhibition of α-glucosidase activity by the tested seaweed products, the IC_{50} values showed variation, and it was 38.9% low for fucoidan compared with the ethanolic extract.

It was reported that the inhibition of α-amylase and α-glucosidase was differently modulated by fucoidan among the seaweed species (18). Fucoidan from *Ascophyllum nodosum* exhibited inhibition against α-amylase; whereas, fucoidan from *Fucus vesiculosus* showed less effect. The level of inhibition by fucoidan against α-amylase activity depends upon its chemical structure in a particular ratio of α-fucose to other monosaccharides, sulfate content, monosaccharide, and uronic acid contents. It was also reported that fucoidan of *Undaria pinnatifida* composed of a repeating backbone structure of alternatively linked α(1-3) and α(1-4) fucose and galactose units, with a high degree of sulfation also exhibited potent inhibitory activity against α-amylase (20). Thus, it is inferred that the backbone structure of fucoidan plays an important role in determining its inhibitory activity against α- amylase (19, 21).

The inhibition effect of fucoidan against α-amylase has been reported to be dependent on its sulfate content (22). A sulfate content of about 51% was required for α-amylase inhibition activity by fucoidan isolated from Korean brown seaweed *U. pinnatifida* and also reported that fucoidan with a sulfate content of 42.0% did not exhibit inhibitory activity against α-amylase. However, in the present study, the fucoidan isolated from *S. wightii* from the southeast coast of India, with an average sulfate content of 24.9 ± 1.2% (w/w) showed a significant inhibitory effect against α-amylase and α-glucosidase. This result is also consistent with the earlier report that fucoidan from New Zealand and *U. pinnatifida* with an average sulfate content of 22.8 ± 1.0% (w/w) exhibited a significant inhibitory effect against α-amylase (20). Thus it is evidenced that seaweed fucoidan inhibits α-amylase and α-glucosidase not only because of sulfate content but also together with other factors or structural futures involved in specific enzyme inhibitory activities of sulfated polysaccharides like fucoidan.

It was also reported that the fucoidan displayed α-glucosidase inhibition via its hydrogen scavenging activity, which is similar to the α-glucosidase inhibition mechanism of most polyphenolic compounds (19). The hydrolysis of α (1-4) glycosidic bond by α-glucosidase requires the presence of hydrogen at the active site of α-glucosidase. Thus, it has been proposed that α-glucosidase inhibitor such as fucoidan scavenges the hydrogen ion at the catalytic site of α-glucosidase thereby inhibiting the enzymatic activity (23). However, an alternate mechanism was proposed which implies that fucoidan may inhibit α-glucosidase activity is by mimicking the enzyme-substrate, similar to the mode of action of acarbose (24).

The results of the present study have shown that fucoidan from *S. wightii* inhibits the α-glucosidase more efficiently, compared with α amylase, and the IC_{50} was low (196.0 µg/mL) for fucoidan. However, the ethanolic extract inhibited α-glucosidase at a minimum level with IC_{50} value 320.7 µg/mL. It was reported that fucoidan from *U. pinnatifida* inhibited α-glucosidase through competitive inhibition by competing with substrate and bind to the active sites of α-glucosidase might be modulated by electrostatic interaction between the negatively charged sulfate group of fucoidan and enzyme (19). This would account for the higher α-glucosidase inhibitory activities of over non-sulfated fucoidan. Thus it is inferred that the conformation and electrostatic changes on the fucoidan molecule may participate in active site binding to inhibit the activity of α-glucosidase (25, 26).

Fucoidan from *U. pinnatifida* inhibited the α-amylase and α-glucosidase by uncompetitive inhibition (20). This implies that fucoidan is bound to the enzyme-substrate complex instead of binding to the enzyme itself (either active site or other binding sites). This is similar to the mechanism of acarbose on α-amylase reported by Oudjeriouat et al (27). It was also reported that acarbose had a much poor affinity to substrates to the α-amylase active sites. As such in the presence of both substrate and inhibitor, the enzyme-substrate complex would be formed at a much higher rate than that of the enzyme-inhibitor complex. The formation of an enzyme-substrate complex, in turn, activated a secondary binding site on α-amylase, for which the inhibitor acarbose had a high affinity, thereby forming the enzyme-substrate inhibitor complex, which inhibited the activity of the enzyme. Therefore, it could be proposed that fucoidan also binds to the secondary binding site on the enzyme that is only functional which the substrate binds to its active site, and thereby inhibits the activity of α-amylase.

Fucoidan isolated from *S.wightii* also inhibited the α-glucosidase by a similar uncompetitive inhibition. In α-glucosidase inhibition, the binding of fucoidan to the secondary binding site of the enzyme could be facilitated by the electrostatic interactions between the negatively charged sulfate group of fucoidan and the secondary binding site of the enzymes. This may also account for the enhanced amyloglucosidase inhibition by fucoidan with higher sulfate content (22). A study on the un-competitive inhibition of fucoidan isolated from *U. pinnatifida* supports the present work (20).

Fucoidan also inhibits the α-glucosidase activity through another possible mechanism of slowing down the diffusion of glucose from the active site of the enzyme as a result of the viscosity of fucoidan (28, 29). The above-highlighted information indicated that fucoidan exerts inhibition on α-amylase and α-glucosidase activities through binding at a secondary site of the enzyme-substrate complex via electrostatic interaction involving sulfate group of fucoidan, as well as by increasing the viscosity of the reaction medium. It was also reported that crude fucoidan isolated from brown seaweeds such as *Sargassum polycystum* and water extract of *Turbinaria conoides* (50 mg extract/3 mL aqueous) showed the highest inhibitory activities on α-amylase and α-glucosidase (30). However, low molecular weight (> 30 KDA) fucoidan from both the seaweed exhibited low inhibitory activities on α-amylase (16.9% and 9.7%), but a high inhibition (99.1% and 66.0%) was noted against the α-glucosidase activity.

Conclusion:

The inhibitory effect on α-amylase and α-glucosidase by the isolated molecule; fucoidan from S. wightii was higher compared to the crude ethanolic extract. Hence, fucoidan from the seaweed *S. wightii* can be used as an adjunct for the treatment of diabetes.

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Conflict of interest:

None to declare

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