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Isolation, characterisation and *in vitro* screening of anticataract potential of Fucoidan from *Sargassum wightii* Greville

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ABSTRACT

Introduction: The present study was designed to isolate Fucoidan from *Sargassum wightii* Greville and to evaluate its *in-vitro* anti cataract potential against galactose induced cataract in isolated goat lenses.

Methods: Fucoidan was isolated from *S. wightii* Greville and its sulfate content was estimated through barium chloride method. Isolated goat lenses were divided into 5 groups as group-I (normal control- vehicle treated), group-II (disease control- galactose treated 55 mM), group-III (galactose 55 mM + standard-ascorbic acid 20 µg/mL), group-IV (galactose 55 mM + Fucoidan-20 µg/mL) and group-V (galactose 55 mM + Fucoidan 40 µg/mL) and incubated for 72 hours, respectively, and subjected to biochemical estimations. The opacity of lenses was also noted prior to biochemical estimation.

Results: The percentage yield of isolated Fucoidan was found to be 0.65%. The extent of sulfation in the isolated Fucoidan was found to be as high as 33%. In high-performance thin-layer chromatography (HPTLC) studies, Peak 3 with Rf value of 0.18 matched with the standard D-glucose Rf value. Galactose induced cataractous lenses showed significant oxidative stress when compared to normal lenses whereas treatment with Fucoidan 20 and 40 µg/mL significantly combated oxidative stress and prevented the development of cataract when compared to cataractous lenses. The results obtained with the treatment of Fucoidan were dose dependant and comparable to standard.

Conclusion: The present study substantiates the claim of anti-cataract potential of Fucoidan, which may be correlated to its anti-oxidant property.

Implication for health policy/practice/research/medical education:
Fucoidan may be utilized in prevention as well as treatment of cataract. Further studies can reveal the clinical significance of Fucoidan in treatment of cataract with a suitable formulation.

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Introduction

Cataract is one of the main reasons for the cause of blindness worldwide. Due to cataract, approximately 19 billion people are visionless in the world (1). It leads to opacification of the eye lens and impairs vision. Alteration in some biochemical processes such as oxidative stress, altered epithelial metabolism, phase transition, calpain-induced proteolysis, calcium accumulation, and cytoskeletal loss are related to the development of cataract (2). Though the cause of cataract is not fully understood, the major mechanism involved in the development of cataract is oxidative damage to the constituents of the eye lens (1).

Cataract is accompanied by alterations in antioxidant system such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) (3). It is estimated that there will be a rise in total number of persons with cataract to 30.1 million worldwide by 2020 (4). World Health Organization (WHO) launched Vision 2020, to eliminate cataract

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as priority diseases (5). Therefore, there is an urge for pharmacological intervention to maintain transparency of the crystalline lens. A large number of herbal drugs such as *Camellia sinensis, Ocimum sanctum,* soybean and lycopene are reported to offer protection against cataracts with their antioxidant properties (4).

Sargassum wightii Greville is one of the important species belonging to the genus *Sargassum*. It is widely distributed in the southern coasts of Tamilnadu, India and many parts of Asia (6). It has been traditionally used in the Chinese medicine and wide range of bioactive properties has been reported from this species (7-13). This genus is an ideal target for investigating presence of biomolecules for various medical and industrial applications (14).

Fucoidan is a sulfated polysaccharide which is commonly found in most of the seaweeds (15). The medicinal benefits of seaweed are mainly due to the presence of sulfated polysaccharides such as Fucoidan (16,17). Recent researches on Fucoidan have been broadly positive in support of its medical benefits (18). The term Fucoidan is generally used to describe the family of polysaccharides such as L-fucose as one of the major monosaccharides, which are highly sulfated and contain varying amounts of galactose, glucuronic acid and xylose and several other major constituents (19).

Fucoidan has recently been reported to possess a wide range of bioactivities (20) including anti-coagulant (21,22), anti-cancer (23-26), anti-inflammatory (27) neuroprotective (28) antioxidant and antibacterial properties (19,29). The effectiveness of these activities is related to the chemical composition of Fucoidan, mainly due to its high degree of sulfation (30,31).

Studies have proved that Fucoidan is non-toxic, non-allergenic, and it has no negative effects on the human body. This statement is further supported by the fact that nutraceutical and food supplements containing Fucoidan have been marketed for several years with no known adverse effects (32).

The aim of the present study was to isolate and characterize Fucoidan from *Sargassum wightii* Greville and to evaluate its *in vitro* anti-cataract potential against galactose induced cataract in isolated goat lenses.

Materials and Methods

Isolation of Fucoidan

Twenty-five grams of coarsely powdered sample of *S. wightii* was taken and 500 mL of ethanol was added. The mixture was subjected to mechanical stirring for 12 hours at room temperature and centrifuged at 2000 rpm for 10 minutes. The mixture was then centrifuged and the residue was dried at room temperature. To 5 g of residue 100 mL of distilled water was added and stirred at 65˚C for 1 hour. Then it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and 1% $CaCl_2$ was added and the mixture was kept at 4˚C overnight to precipitate alginic acid. The solution was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected. To the supernatant, ethanol 99% was added to attain the concentration of 30% and kept 4˚C for 4 hours. Further the solution was centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected. To the supernatant, ethanol 99% was added to attain the concentration of 70% ethanol and kept at 4˚C overnight. Fucoidan was obtained through filtration and percentage yield was calculated (33,34).

Determination of sulfate content in Fucoidan

Sulfate content of isolated Fucoidan was determined using $BaCl_2$. Conditioning reagent was prepared adding 50 mL glycerol, 30 mL of HCl, 300 mL of deionised water, 100 mL of isopropyl alcohol and NaCl, stirring mechanically overnight. Fifteen milligrams of dried Fucoidan was taken in separate test tubes containing 5 mL of 4M HCl and subjected to hydrolysis at 100˚C for 2 hours. Potassium sulfate in the range of 200-1000 µg/mL was used as standard. To the hydrolysed sample solution, 15 mL of deionised water was added followed by 5 mL of conditioning reagent. Then the mixture was subjected to mechanical stirring at constant speed. Further 0.3 g of $BaCl_2$ was added and stirred for 1 minute and the mixture was allowed to stand for 4-6 minutes for precipitation of $BaSO_4$. The absorbance was measured at 420 nm using water as blank (32).

HPTLC studies

Fucoidan (30 mg/mL) was taken and hydrolysed with 6M trifluoroacetic acid at 100˚C for 24 hours. Centrifugation of hydrolysate was done at 7380 rpm for 10 minutes and obtained supernatant was used for high-performance thin-layer chromatography (HPTLC) studies. Fifty milliliters of n-butyl alcohol/acetic acid/water (6:3:1) was used as solvent system. D-glucose (standard – monosaccharide) was used as standard (35).

Four milliliters of the standard and test solution were loaded as 6 mm band length in the 5 x 10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plates were kept in TLC twin trough developing chamber and developed in the respective mobile phase up to 90 mm. The developed plate was sprayed with respective spray reagent (5% sulphuric acid in methanol) and heated until the spot appeared. The plate was photo-documented at day light and UV 425 nm using CAMAG REPROSTAR 3 chamber. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in CAMAG REPROSTAR 3 and captured the images at white light (425 nm).

The details of HPTLC were as follows: Plate: Aluminium plate pre-coated with silica gel GF254; Thickness: 0.2 mm plate size: 5 × 10 cm; Sample application: 4.0 µL; Solvent

system: n-butyl alcohol: glacial acetic acid: water (6: 3: 1); Detection: UV (425 nm); Instrument: CAMAG TLC Scanner 3 and LINOMAT- V. CAMAG TLC Scanner 3 and LINOMAT-V densitometric evaluation system with WINCATS software was used for scanning of thin layer chromatogram objects in reflectance or transmission mode by absorbance 425 nm respectively (36).

Anti-cataract activity

Experimental design

The anti-cataract potential of Fucoidan was evaluated in isolated goat lenses. The fresh goat lenses were procured from local slaughter house. Thirty lenses (Figure 1) were divided into 5 groups with 6 lenses in each group (Figure 2). For inducing cataract, the goat lenses were incubated with Galactose (55 mM) for 72 hours (15). Opacification of lenses were evaluated on a graph paper and counting number of squares visible through the lenses macroscopically (3).

The lenses were grouped as:

- Group 1 - Normal control (vehicle treated)
- Group 2 - Disease control (galactose 55 mM treated cataractous lenses)
- Group 3 - Standard drug treated (galactose 55 mM + ascorbic acid-20 µg/mL)
- Group 4 - Fucoidan low dose treated (galactose 55 mM + 20 µg/mL)
- Group 5 - Fucoidan high dose treated (galactose 55 mM + 40 µg/mL)

Vehicle treated non-cataractous lenses were used as normal control (group-I). In group II, cataract was induced by incubating the lenses with galactose 55 mM for 72 hours, which served as positive control. Group-III was incubated simultaneously with galactose 55 mM and standard drug ascorbic acid. Group IV and V were incubated simultaneously with galactose 55 mM and Fucoidan 20, 40 µg/mL, respectively, for 72 hours.

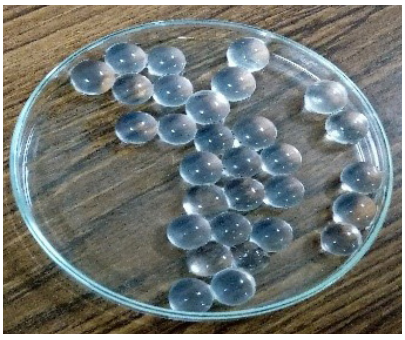

Figure 1. Isolated goat lenses.

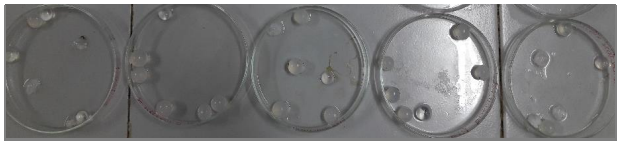

Figure 2. Grouping of lenses with 6 lenses in each group.

The dose range of Fucoidan was chosen after a pilot study conducted with the range of 10–80 µg/mL. Streptomycin was added to all groups of lenses to avoid any microbial contamination during incubation.

After incubation, the lenses were visualised and their degree of opacity was noted. For MDA estimation, the homogenate was prepared using 10% (w/v) 0.1 M Tris–HCl buffer (pH 7.5). For the estimation of GSH, catalase, total protein and aldose reductase inhibition % activity, the lenses were homogenized with 0.1M potassium phosphate buffer to 10 volumes and centrifuged at 4000 rpm for 15 minutes. The supernatant was used for biochemical estimations (37).

Biochemical parameters

(a) Estimation of malondialdehyde level

One milliliter of the homogenate was combined with 2 mL of TCA–TBA–HCl reagent (15% trichloroacetic acid and 0.375% thiobarbituric acid in 0.25 N HCl) and boiled for 15 minutes. The mixture was then cooled and the precipitate was removed after centrifugation at 1000 rpm for 10 minutes. The absorbance was measured at 535 nm against a blank without tissue homogenate. The values were expressed as nmole/100 mg of tissue (2).

(b) Estimation of reduced glutathione level

One milliliter of 10% homogenate was mixed with 1 ml of 5% TCA (w/v) and the mixture was allowed to stand for 30 minutes and centrifuged at 2500 rpm for 15 minutes. 0.5 mL of supernatant was taken and 2.5 mL of 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was added and mixed thoroughly. The absorbance was recorded at 412 nm and the results were expressed as nmole/100 mg tissue (1).

(c) Estimation of catalase activity

To 0.1 mL of supernatant, 1 mL of phosphate buffer and 0.4 mL water was added. Reaction was started by adding 0.5 mL $H_2O_2$and the mixture was incubated at 37°C for 1 minute. Reaction was stopped by adding 2 mL of dichromate: acetic acid reagent and kept in boiling water bath for 15 minutes. The mixture was cooled and the absorbance was read at 570 nm before and after reaction. CAT activity was calculated in terms of U/mg of tissue (38).

(d) Estimation of total protein content

Chemicals required: Reagent I: 48 mL of 2% Na_2CO_3 in 0.1 N NaOH, 1 mL of 1% sodium potassium tartrate in H_2O, 1 mL 0.5% $CuSO_4$ in H_2O; Reagent II- 1-Part Folin-Phenol [2 N]: 1- Part water; Bovine Serum Albumin (BSA) standard - 1 mg/mL. 0.2 mL of BSA was taken in 5 test tubes and volume was made up to 1 mL using distilled water. Then, 0.2 mL of lens homogenate was taken in a test tube and volume was made using 1 mL distilled water. The test tube

with 1 mL distilled water was served as blank. 4.5 mL of reagent I was added and incubated for 10 minutes. After incubation, 0.5 mL of reagent II was added and incubated for 30 minutes. Absorbance was measured at 660 nm and a standard graph was plotted, then, the amount of protein present in the given sample was estimated from the standard graph and expressed as mg/dL (39).

(e) Estimation of aldose reductase inhibition activity %

To 0.1 mL of lens homogenate, 0.7 mL of phosphate buffer (0.067M), 0.1 mL of NADPH and 0.1 mL of D L-glyceraldehyde (substrate) were added. By addition of the substrate, the enzymatic reaction was started. The absorbance was recorded at 340 nm for at least 3 minutes at 30 seconds intervals using UV spectrophotometer against blank (without the substrate DL- glyceraldehyde). Aldose Reductase (AR) inhibitory activity was expressed as % inhibition activity (40).

Statistical analysis

Data were expressed as mean ± SEM (n = 6). Significant difference between groups was determined using one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered as significant.

Results

The percentage yield of isolated Fucoidan was found to be 0.65%. The sulfate content in the isolated Fucoidan as per barium chloride method was found to be 33%. HPTLC studies showed various peaks of fucoidan hydrolysates (Figure 3). One of the hydrolysates of Fucoidan with Rf value 0.18 (Peak 3) matched with the standard D-glucose Rf value (Figure 4). This confirms the presence of D-glucose in the isolated Fucoidan.

Photographic study of lenses

Normal lens incubated with artificial aqueous humour showed complete transparency (Figure 5A). The lenses which were treated with galactose 55 mM showed complete opacification (Figure 5B) of the lens when compared with normal lenses (Figure 5A). Lenses incubated with galactose 55 mM and ascorbic acid (20 µg/mL) showed almost normal transparency (Figure 5C) when compared with cataractous lenses (Figure 5B). The lenses incubated simultaneously with galactose 55 mM and Fucoidan at a concentration of 20 µg/mL showed relatively less opacity (Figure 5D) when compared with cataractous lenses (Figure 5B). The lenses incubated with galactose 55 mM and Fucoidan at a concentration of 40 µg/mL showed almost normal transparency (Figure 5E) when compared to the cataractous lenses (Figure 5B) and the transparency was also found to be comparable to the ascorbic acid treated lenses (Figure 5C).

Study of anti-cataract potential of Fucoidan

Table 1 shows the anti-cataract potential of Fucoidan.

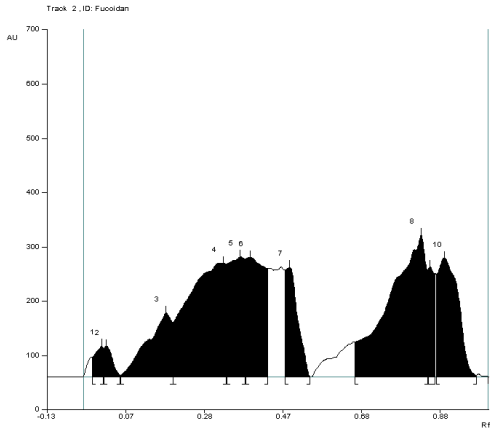

Figure 3. HPTLC Profile of hydrolysates of Fucoidan.

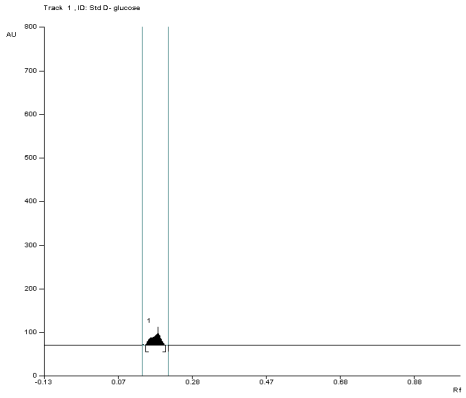

Figure 4. HPTLC profile of standard D-glucose.

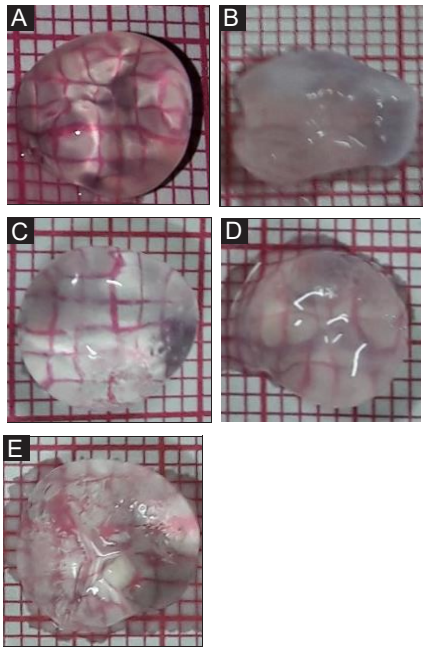

Figure 5. (A) Normal control; (B) Disease Control; (C): Ascorbic acid 20 µg/mL treated; (D): Fucoidan 20 µg/mL treated; (E): Fucoidan 40 µg/mL treated

Normal lenses contain high amount of protein, aldose reductase inhibition activity % and catalase activity compared to cataractous lenses. The MDA was found to be the least in the normal lenses (normal control) and highest in the cataractous lenses (disease control). The lenses treated with Fucoidan 20 and 40 µg/mL, ascorbic acid 20 µg/mL (standard) restored the level of protein, aldose reductase inhibition and catalase activity whereas, reduced the level of MDA.

Effect of Fucoidan on lens MDA

There was a statistically significant increase in the level of malondialdehyde in the cataractous lenses (disease control) when compared with the normal lenses (normal control). Treatment with Fucoidan 20 and 40 µg/mL, ascorbic acid-20 µg/mL showed significant reduction ($P<0.001$) in malondialdehyde level when compared with cataractous lenses (disease control).

Effect of Fucoidan on lens GSH level

A statistically significant reduction in GSH level was observed in galactose 55 mM treated cataractous lenses (disease control). Lenses incubated simultaneously with galactose 55 mM and Fucoidan 20 µg/mL showed a significant increase ($P<0.01$) in the level of GSH when compared with cataractous lenses. Lenses which were treated with galactose 55 mM and Fucoidan 40 µg/mL simultaneously showed a significant increase ($P<0.001$) in the level of GSH and it was found to be comparable to ascorbic acid treated lenses (standard).

Effect of Fucoidan on lens catalase activity

There was a statistically significant decrease in catalase activity observed in cataractous lenses (disease control) when compared with normal lenses (normal control). Treatment with Fucoidan 20 and 40 µg/mL showed highly significant increase ($P<0.001$) when compared with cataractous lenses (disease control). The results were comparable to the standard group.

Effect of Fucoidan on total protein content

A statistically significant reduction was observed in the level of total protein in cataractous lenses (disease control) when compared with normal lenses (normal control). Treatment with Fucoidan-20 and 40 µg/mL showed significant increase with $P<0.01$ and $P<0.05$, respectively. Treatment with ascorbic acid-20 µg/mL showed a significant increase ($P<0.001$) in the level of total protein when compared with cataractous lenses (disease control).

Effect of Fucoidan on aldose reductase inhibition activity %

There was a statistically significant decrease in aldose reductase inhibition activity % in the cataractous lenses (disease control) when compared with normal lenses (normal control). Lenses which were incubated with Fucoidan 20 and 40 µg/mL showed significant increase ($P<0.01$) in aldose reductase inhibition activity %. Treatment with ascorbic acid (standard) showed highly significant increase ($P<0.001$) in aldose reductase inhibition activity % when compared with cataractous lenses (disease control).

Discussion

Oxidative stress is known to be a common basic mechanism of cataractogenesis. Various studies reported the potential of antioxidant agents against cataractogenesis in experimental models of cataract. Augmentation of anti-oxidant defences of the lens has been reported to prevent or delay cataract. Galactose induced cataract is the most commonly used experimental model. The galactose produces a large amount of its reduced form, galactitol inside the lens that leads to osmotic stress (41).

The molecular mechanisms which are involved in the development of cataract are mainly non-enzymatic glycation of eye lens proteins, oxidative stress and activated polyol pathway which accelerates the generation of reactive oxygen species. The produced reactive oxygen species enhance chemical modification of proteins in the lens (40). Lipid peroxidation and other oxidative degradative processes are the common cause of genesis of free radicals and the lens becomes vulnerable to oxidative stress. MDA is one of the end products of lipid peroxidation which is reported to have major role in the development of cataract. Its role in cataractogenesis is due to its cross-linking ability. The accumulation of oxidised residues in lens proteins and enzymes interfere in normal metabolic process which results in disruption of intracellular protein matrix and subsequent loss of lens transparency (42). In the present study, cataractous lenses

Table. 1. Anticataract activity of Fucoidan on isolated goat lenses

Groups	MDA (nmoles/100 mg)	GSH (nmoles/100 mg)	Catalase activity (U/mg of tissue)	Total protein content (mg/dL)	Aldose reductase inhibition activity %
Normal control	2.45±0.07	52.75±0.84	5.39±0.38	26.86±1.04	25.58±1.14
Disease control	6.39±0.48 [a]	35.73±1.54 [a]	3.67±0.39 [a]	18.19±0.34[a]	19.95±0.68 [a]
Ascorbic acid 20 µg/mL	2.64±0.21[b]	55.46±1.23[b]	6.24±0.38[b]	27.1±1.99[b]	28.3±1.69[b]
Fucoidan 20 µg/mL	3.28±0.18[b]	43.22±1.00[c]	4.90±0.56[b]	23.39±0.45[d]	27.24±1.92[c]
Fucoidan 40 µg/mL	2.04±0.17[b]	53.90±1.34[b]	6.04±0.55[b]	24.67±0.43[c]	27.21±0.63[c]

All values are expressed as mean ± SEM; n=6; [a] $P<0.001$ when compared to normal control. [b] $P<0.001$ when compared to disease control, [c] $P<0.01$ when compared to disease control, [c] $P<0.05$ when compared to disease control.

were observed to have high levels of MDA suggesting ample lipid peroxidation and oxidative stress. Treatment with Fucoidan 20 and 40 µg/mL and ascorbic acid-20 µg/mL showed significant reduction in the MDA level which indicates the anti-oxidant potential of Fucoidan.

Oxidative stress leads to denaturation and aggregation of lens proteins which are further observed by lens opalescence. CAT and GSH are the important components of innate enzymatic defences of the lens. Detoxification of significant amounts of hydrogen peroxide is the main role of catalase and thereby prevents damage to the membrane and biological structures. GSH is reported to preserve the integrity of phospholipid bilayer membrane by inhibiting lipid peroxidation (3). In the present study, cataractous lenses were observed to have high opacity compared to normal lenses. They also showed significant reduction in the level of total protein, GSH, catalase activity and aldose reductase % inhibition. These results are postulated to occur as a result of free radicals formation and increased oxidative stress. The level of protein, GSH and catalase activity was restored with the treatment of Fucoidan 20 and 40 µg/mL when compared to the cataractous lenses. The results obtained with the treatment of Fucoidan were dose dependant and comparable with the ascorbic acid 20 µg/mL treated lenses.

Aldose reductase is considered to be the main factor responsible for significant production of polyol (sugar-induced cataractogenesis) and its accumulation in the lens which leads to the development of cataract. Accumulation of polyols increases intra-cellular ionic strength in the lens that leads to excessive hydration and subsequent loss of membrane integrity (43). In the present study, cataract lenses showed high activity of aldose reductase which could be correlated to polyol accumulation. Treatment with Fucoidan 20 and 40 µg/mL significantly decreased Aldose reductase activity and reversed the polyol accumulation.

Conclusion

From the above results, it is concluded that Fucoidan exhibited potential anti-cataract activity against galactose induced cataract in isolated goat lenses. *In vitro* study may not directly correlate with the *in vivo* conditions therefore, *in vivo* studies in animal models are needed for further elucidation. However, the present study substantiates the claim of anti-cataract potential of Fucoidan, which may be correlated to its anti-oxidant property.

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Authors' contributions

SR generated the idea, SAM, KK, PPK and KS executed the studies in accordance with the designed procedure and collected data under the supervision of SR. SR, GR performed data analysis. SAM and KK prepared the initial draft. SR revised the draft and produced final manuscript with the consent of GR. All are well concerned about the latest version and agreed for the publication.

Conflicts of Interest

The authors declare there is no conflict of interest in the study.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission redundancy) have been completely observed by the authors.

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