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Screening for Antimicrobial Efficacy of Fucoidan Isolated and Characterized from the Brown Seaweed *Stoechospermum marginatum* (C. Agardh) Kutzing Collected along the Coastline of Tamil Nadu

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ABSTRACT: The need for novel antibacterial biomolecules has been a focus for many years. The major challenge is the designing of synthetic molecular drug targets that are not prone to rapid resistance development. The current study is designed for the antibacterial screening efficacy of seaweed fucoidan. Fucoidan are sulfated polysaccharides derived from brown seaweeds with diversified pharmacological activities. The brown seaweed *Stoechospermum marginatum* is collected from the southern coastline of Tamil Nadu. The structural components of the extracted fucoidan from the seaweed were identified and confirmed by performing a specific test for the presence of total carbohydrate, L-fucose, and sulfate content using biochemical methods. HPLC and FTIR analysis confirmed the structural entity of fucoidan. It showed that the extracted fucoidan contains fucose and sulphate. Fucoidan were evaluated for their antibacterial capability against clinical pathogens. The antibacterial efficacy of fucoidan was performed against bacterial clinical pathogens using agar well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) methods. The maximum antibacterial activity of 18.14 ± 0.37 mm was derived for gram-negative Klebsiella pneumonia and the minimum activity was noticed as 10.6 ± 0.18 for gram-positive Streptococcus faecalis. Fucoidan are found to be effective against both strains of bacteria and found effective against gram-negative strains. The seaweed fucoidan can be utilized as a lead molecule for combating lethal bacterial diseases.

Keyword: Seaweeds, fucoidan, sulfated polysaccharides, fucose, antibacterial.

INTRODUCTION

Brown seaweeds belonging to the family Dictyoceae are a rich source of sulfated L-fucose called sulfated polysaccharides. They were called fucoidan because they are the first sulfated polysaccharides derived from the order Fucales which belongs to the Phaeophyceae family of algal species. The fucoidan are found embedded in the intercellular thallus tissues. They were proved to showcase the presence of bioactive principles which are effective against different viral species. It has shown anti-ulcer and anti-adhesive, anticoagulant, anti-inflammatory and antiproliferative, and antitumor properties (Eluvakkal *et al*., 2010).

Discovering and developing acceptable novel antibiotics is challenging and tricky. Moreover, the research and development process is expensive and time-consuming and often leads to failures. Research report reveals that vascular smooth muscle cells are inhibited by fucoidan and it blocks the proliferation and binding of sperm-ova binding in various species (Berteau *et al.*, 2003). Herbal biomolecules are proven antibacterial agents with promising antibacterial activity (Shyamala Chandra Rokkala *et al.*, 2023).

People will be sicker for longer, people will have fewer options to get better, and the cost of care will increase. In the future, more people may also die from secondary bacterial infections that are resistant to antibiotics.

Different seaweed species exhibit structurally different polysaccharide and it varies with the different parts of the thallus. Brown seaweeds are rich sources of cell wall polysaccharides called fucoidan (Zhang 2016). Individual units of L-fucose and sulfate ester groups are the structural units of fucoidan (Bilan *et al.*, 2006) and found to have a broad range of pharmacological and biomedical applications (Guven *et al.*, 1999). The presence of sulfate moiety in its chemical structures proves to be a potent antimicrobial agent (Berteau *et al.*, 2003). The presence of high sulfate content in the different species shows profound antiviral and antimicrobial activity (Adhikari *et al.*, 2006). Hence isolation of novel fucoidan from brown seaweed *S. marginatum* will serve as potent biomolecules with

different pharmacological activity. Extraction, isolation and characterization of fucoidan from seaweeds are still in the dormant stage in India, in spite of abundant number of seaweeds occurring in the Indian coastal line. Brown seaweeds of Indian origin were not studied in an elaborative manner. There were approximately around 21 islands located in the Gulf of Mannar in the southeast coast of India which are protected and declared as a biosphere reserve and seaweeds (Saravanan *et al.*, 2006). The biological potential of seaweeds are still unexploited completely, there is a lag and lacuna in the utilization of seaweed fucoidan. The antimicrobial fucoidan derived from seaweed may arrest the growth of bacteria, fungi, viruses, and protozoa by different mechanisms than those of presently used antimicrobials and may have a promising clinical value in the treatment of resistant pathogenic microbial strains.

Hence the proposed study was extensively focused on isolation, characterization, and identification sulfated fucoidan from the brown seaweed *S. marginatum* by using modern analytical tools like RP-HPLC and FTIR and subjecting the isolated fucoidan for their efficacy against clinically pathogenic bacterial strains.

MATERIALS AND METHODS

Seaweed Profile

Brown seaweed *S. marginatum* was widely distributed from in the Indian coastal region of the Bay of Bengal. It is found rich in the southern coastal region of Tamil Nadu near the Tuticorin seashore. *S. marginatum* is a flat and thick thallus, found branched without a midrib. It possessed an entire margin, bifid, flat and truncated apex. The branches are notched at the apex. It grows rigorously up to a length of 40 cm. The mature seaweed can be identified by the presence of dark lines around the sporangia. The morphological features of the seaweeds were the presence of Small Square shaped darkly stained, thick, and cutinized epidermal layers. The epidermal layer was composed of many layers of identical parenchymatous cells. Vertical elongated cells are unique features of the stalk of the thallus (Kumaresan *et al.*, 2008). The seaweed biomass was abundant during the month of April to May of every year in the southern coast of Tamil Nadu.

Sampling of seaweed S. marginatum

Fully grown, healthy, and contamination free brown seaweed *S. marginatum* (C.Agardh.) Kutzing found along the southern coastline of, Tamil Nadu, India was collected during the month of March–April 2019. The collected seaweed was subjected to initial seawater washing and were submerged in a running tap to get rid of adhering epiphytes, sand and other unwanted materials. The seaweed was dried initially under the sun for an hour and then under the shade for 3 days. The dried *S. marginatum* seaweed sample was pulverized into a fine powder and used for the extraction of crude fucoidan by adopting two different methods as described by Chotigeat *et al.* (2004). The evaluation of total carbohydrates was analysed as per the methods described by Dubois *et al.*, 1956. Evaluation of L-fucose (Dische *et al.*, 1948) and the presence of sulphate were confirmed by comparison with the reference compounds (Sivakumar and Arunkumar 2009). Percentage dry weight is used as a term for expressing the Fucoidan yield and the Total carbohydrates, L-fucose and sulphate were estimated from the dry crude fucoidan was expressed in percentage dry weight of crude fucoidan isolated from *S. marginatum.*

Extraction of Crude Fucoidan

Acid extraction of fucoidan

Accurately weighed 100 g of powdered seaweed was soaked in 100 mL of 0.1 N HCl at 95ºC for 12 h. This procedure was repeated thrice and combined extracts were filtered using Whatman No.1 filter paper. The filtrate was dialyzed against water and lyophilized to dryness. Determination of true fucoidan done by gravimetric method and expressed on the basis of algal dry weight. Seaweed dry weight was calculated by keeping specified quantity of fresh seaweed which was dried in an oven at 60ºC until it attains constant weight (Chotigeat *et al.*, 2004).

Characterisation of Fucoidan

Estimation of the composition of crude fucoidan

The biochemical composition of Crude fucoidan was derived by analyzing the total carbohydrates, L-fucose, and sulphate contents.

Estimation of total carbohydrate:

5 % Phenol solution

Five grams of phenol was dissolved and made up to 100 mL in distilled water and stored at room temperature.

Conc. Sulphuric acid (H_2SO_4).

Concentrated H_2SO_4 acts as a reaction catalyst in the preparation of phenol sulfuric acid reagent.

Procedure

100 mg dried crude fucoidan was ground as fine powder using a mortar and pestle with 10 ml of distilled H_2O and kept the volume for 30 minutes with intermittent grinding to obtain a homogenate solution. To an aliquot of 200ml of the in a test tube, 200ml of 5% phenol and 1 mL of Conc. H_2SO_4 were added. Rapid addition of conc. H_2SO_4 promoted the mixing and heat development that was necessary for the assay. The reaction mixture was placed in a dark place for around 30 minutes and the absorbance was measured at 485 nm against blank. The amount of total carbohydrate was determined from a standard graph prepared with different concentrations of D-galactose (Dubois *et al.*, 1951).

Estimation of L-fucose

Reagents

5% Cysteine hydrochloride solution:

Five grams of cysteine hydrochloride was dissolved and made up to 100 mL in distilled H_2O and stored at room temperature.

Sulphuric acid (H_2SO_4) reagent: Prepared by mixing concentrated H_2SO_4 and water in the ratio of 6:1.

Procedure

Cysteine-sulphuric acid method is used to evaluate L-fucose (Dische *et al.,* 1948). To 1 mL of sample solution in a test tube which was previously chilled in ice water bath, followed by addition of 4.5 ml of Sulphuric acid reagent and mixed thoroughly. The tube was placed in water bath maintained at 25ºC for 3-4 minutes, and subsequently for 3 minutes in a boiling water bath.

In the final step of the evaluation, the test tubes were cooled at room temperature and 0.1 ml of 5% cysteine hydrochloride solution was added to all the test tubes and mixed thoroughly. The absorbance of the sample was measured at 396 nm and 427 nm against reagent blank. Following equation were used to calculate the absorbance values

$$Absorbance = (A396 - A427)$$

Estimation of Sulphate by Turbidometric Method

The sulphate content of the fucoidan was determined by measuring the turbidity using a turbidity meter.

Reagents

(i) 6 M Hydrochloric acid
(ii) 1 N Hydrochloric acid
(iii) 70% Sorbitol
(iv) Barium chloride

Procedure

10 mg of crude fucoidan extracted from *S. marginatum* sample was homogenized with 6 ml of 1 N HCl and transferred to a hydrolyzing tube with proper sealing. The sample is hydrolysed by boiling in a water bath for a period of 4 h. The sample was then cooled to room temperature. It is filtered securely, and the filtrate was diluted to 25ml with distilled water to get the final volume. Transfer 10 ml of the filtered sample solution; add 1 ml of 6 M HCl and 5 ml of 70% sorbitol. The sample was then stirred with a magnetic stirrer while stirring 1 g of barium chloride crystals was added and read at 470 nm within one minute.

Chromatographic Techniques

Chromatographic analysis was performed using conventional thin layer chromatography and modern reversed phase high performance liquid chromatography (RP-HPLC).

TLC analysis

Thin Layer Chromatography analysis was performed on precoated 10 × 20 cm silica gel- G plates (Merck, Germany). The polysaccharides present in *S. marginatum* were qualitatively evaluated and identified. Separation of polysaccharides were done using methanol and water in the 9:1 and Barfode's reagent when sprayed on developed plates showed the appearance of a red-coloured spot confirmed the presence of polysaccharides.

HPLC analysis

The *S. marginatum* extract was centrifuged at 3000 rpm for 10 min and filtered. Isolation of individual sugar moieties was performed on a Shimadzu LC-10AT VP HPLC system, with LC-10AT pump, SPD-10AT UV-Vis detector, Rheodyne injector fitted with a 20 µL injection loop and manual injector. The HPLC system was equipped with a hypersil Octadecysilane (ODS) C-18 column (4.6 × 250 mm, 5µm size) with a guard column supported with C-18. The column was eluted with the gradient elution technique with the elution was carried out with a gradient C-18 flow rate at a temperature in the range of (25-28°C). The mobile phase for elution was composed of methanol (0.1% v/v methanol) (solvent A) and water (solvent B).

The mobile phase was filtered through a 0.45 µm membrane filter and degassed by using a sonicator for 20 minutes to remove the dissolved gasses. A sample volume of 20 µL was injected through the loop and the running time was set to a maximum of 15 min. The detector wavelength of the UV-254 nm. The sample was filtered through Whatmann filter paper No.1 and preserved. The isolated fucoidan were subjected to hydrolysis into their respective monosaccharides using 2 mol/L trifluoroacetic acids. A gradient elution technique was used to isolate the monosaccharide by reverse-phase HPLC and monitored by ultraviolet detection at 245 nm. Chromatographic conditions were depicted in Table 3.

Fourier Transform Infrared Spectrometry (FTIR) Analysis

The isolated fucoidan sample (2 mg) was dried in a desiccator for 48 h, mixed with the IR grade potassium bromide (KBr) powder, ground, and pressed into 1 mm pellets for FT-IR measurement (Perkin Elmer USA, Spectrum 100). The scanning range for the IR spectrum was within the range from 400 to 4000 cm^{-1} (Wang *et al*., 2008).

Antibacterial Assays

Bacterial culture

In the current study, pathogenic bacteria such as *Staphylococcus aureus, Streptococcus faecalis*, *Escherichia coli*, and *Klebsiella aerogenes* were used to screen the antibacterial activity of fucoidan from *S. marginatum*.

Table 1: Chromatographic conditions for separation of fucoidan from *S. marginatum.*

Chromatographic	Specifications
Instrument	Shimadzu LC-10AT VP HPLC
Flow rate	1 mL min^{-1}
Column	Hypersil ODS C-18 column with a C-
Mobile phase	0.1% v/v Methanol in Water
Injection volume	20 µL
Elution	Gradient Elution
Detector	SPD-10AT UV-Vis detector
Detector wavelength	254 nm
Retention time	4.430

Table 2: FTIR spectral characterization of fucoidan in *S. marginatum.*

Peak values	Functional groups
3400 and 2900 cm^{-1}	O-H and C-H stretching
1680 and 1650 cm^{-1}	Carbonyl groups
1240-1260 cm^{-1}	Sulfate groups
810~840 cm^{-1}	C-O-S bending

Table 3: Screening of anti-bacterial activity of fucoidan against human bacterial pathogens

Bacterial pathogens	Fucoidan (500 µg/ml)	Positive control (Ciprofloxacin) (500 µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Staphylococcus aureus	12.06± 0.2	22 ± 0.4	125	250
Streptococcus faecalis	10.6 ± 0.18	16 ± 0.42	250	500
Escherichia coli	15.62 ± 0.25	20 ± 0.28	125	250
Klebsiella pneumonia	18.14 ± 0.37	21 ± 0.22	62.5	150

Antibacterial screening of fucoidan

Screening of antibacterial potency of isolated fucoidan was performed evaluation was performed using agar plate diffusion assay. Muller Hinton's agar medium was prepared, and culture plates were prepared in sterilized water. It is followed by the addition of 0.1 ml of test organisms and swabbed on agar medium by using sterilized loops.

Four 6 mm wells were made on the inoculated plates. Fucoidan with the concentration of 50 µg/ml was prepared. Ciprofloxacin with a concentration of 50 µg/ml was used as a positive control. Sample and positive control were added to the respective wells.

The sample prepared without fucoidan was used as a negative control to check the antibacterial potential. Then the plates were incubated at 37°C for 24 h. The zone of inhibition (in mm) was measured to screen the antibacterial efficacy of the isolated fucoidan.

Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration (MIC) was tested in the listed strains. Equal volumes of each bacterial strain culture were applied to Muller-Hinton's broth (MHB) with different concentrations of fucoidan in the test tubes ranging from 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95, and 0.97 µg/ml, respectively. Ciprofloxacin was used as positive control, whereas, the negative control used for the study was prepared without fucoidan.

These serially diluted cultures were then incubated at 37°C for 24 h. After the incubation period, turbidity was observed. MIC was defined as the lowest concentration of fucoidan that completely inhibited the visible growth of the test microorganisms.

Determination of minimum bactericidal concentration (MBC)

Muller Hinton's agar plates were prepared using sterile plates. Aliquots of serially diluted samples were inoculated and incubated at 37°C for 18 h on the plates previously inoculated with the bacterial strains. The plate inoculated with minimum bactericidal concentration that exhibits no growth was identified as the MBC value for isolated fucoidan.

RESULTS AND DISCUSSION

Thin Layer Chromatography Analysis

The fucoidan polysaccharides present in *S. marginatum* were qualitatively evaluated and identified. Developed plates when sprayed with Barfode's reagent it developed the appearance of a red coloured spot confirming the presence of polysaccharides.

RP-HPLC Analysis

Fucoidan isolated from alcoholic extracts of *S. marginatum* when qualitatively screened for HPLC fingerprinting at a wavelength of 254 nm, the extracts showed a sharp peak with a retention time of (R_T) 4.430 mnts (Fig. 2). The presence of sulfated polysaccharide was confirmed by comparing with the reported standard reference fucoidan.

FT-IR Spectroscopy Analysis of Fucoidan Fractions

The fucoidan extract of *S. marginatum* when subjected to FTIR spectral analysis exhibited the identical vibration bands of fucoidan (Fig. 3). Appearance of the broad peak at around 3400 cm^{-1} was clearly evident of the presence of O-H stretching of alcoholic groups of polysaccharides. The peak at 900 cm^{-1} is the proof of alkyl C-H stretching vibrations. The presence of carbonyl moiety was confirmed when correlating the absorption peak observed at around 1680 to 1650 cm^{-1}. Absorption bands that appeared between 1240- 1260 cm^{-1} confirm the presence of sulphate groups and are considered to be the characteristic functional group peak of fucoidan. The spectrum displayed the C-O-S bending vibration which is specific for sulphate with absorption bands at 810~840 cm^{-1}. An absorption band at 800 cm^{-1} alongside of other prominent bands indicates that all the sulphate groups are bonded to the 2 and 3 positions and a minor part of sulphate was attached to the 4th position of the fucopyranose molecules (Table 2) (Bilan *et al*., 2002).

Fig. 1. Thallus of *Stoechospermum marginatum*

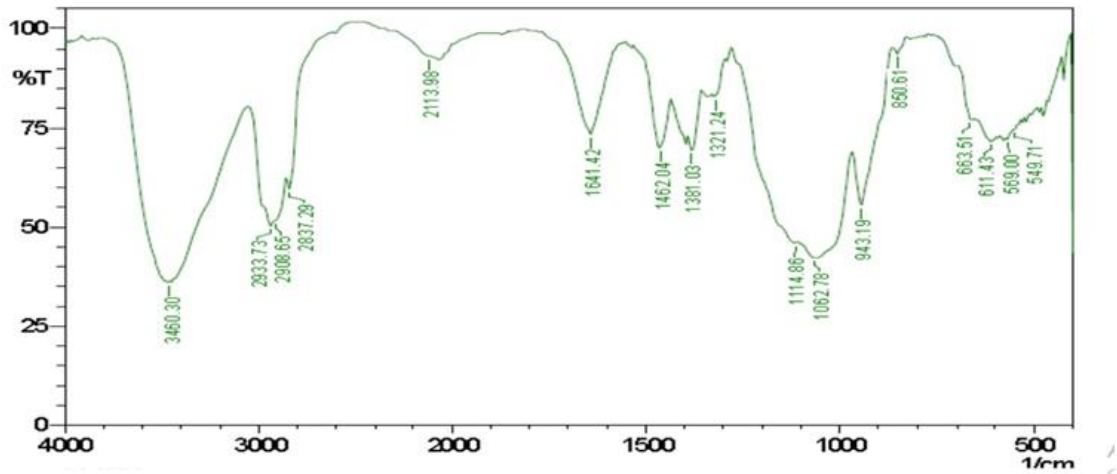

Fig. 2. FTIR Spectrum of Fucoidan from *S. marginatum*

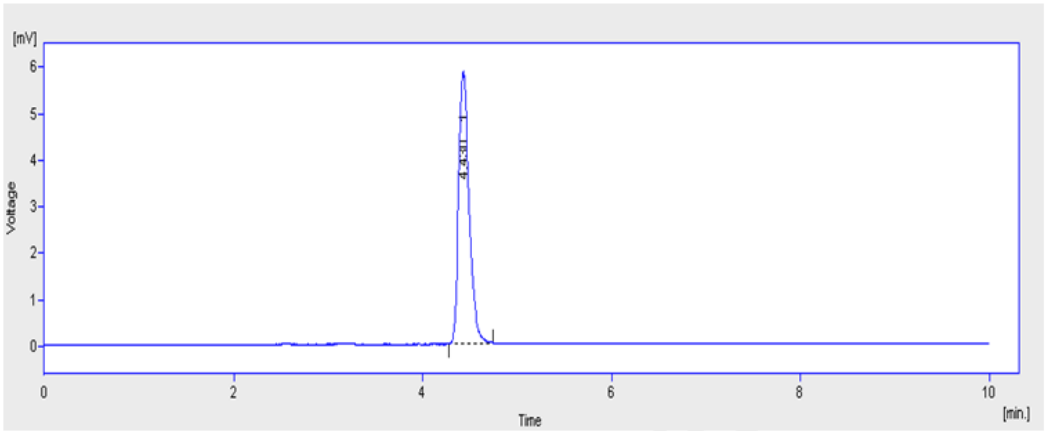

Fig. 3. RP-HPLC Chromatogram of Fucoidan isolated from *S. marginatum*.

Antibacterial Activity

The bacteriostatic and bactericidal effect of isolated fucoidan was screened using cup and plate agar well diffusion method. The fucoidan were subjected to the evaluation of MBC and MIC determinations to access the antibacterial potency and it is displayed in Table 3. The Fucoidan showed maximum antibacterial activity against *K. pneumoniae* (18.14 ± 0.36 mm), and minimum activity against *S. faecalis* (10.6 ± 0.18 mm) (Fig. 4). The minimum inhibitory concentration (MIC) was in the range of 62.5 to 250 µg /ml and the minimum bactericidal concentration (MBC) of fucoidan were between 150 to 500 µg /ml against respective clinical pathogens of bacterial origin. In the pathogens recent study, considerable activity was observed to the bacterial pathogens as mentioned in Table 3.

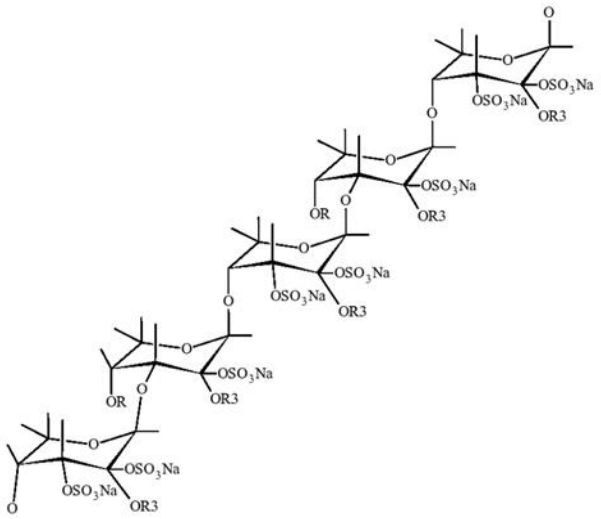

Fig. 4. Structure of Fucoidan isolated from *S. marginatum.*

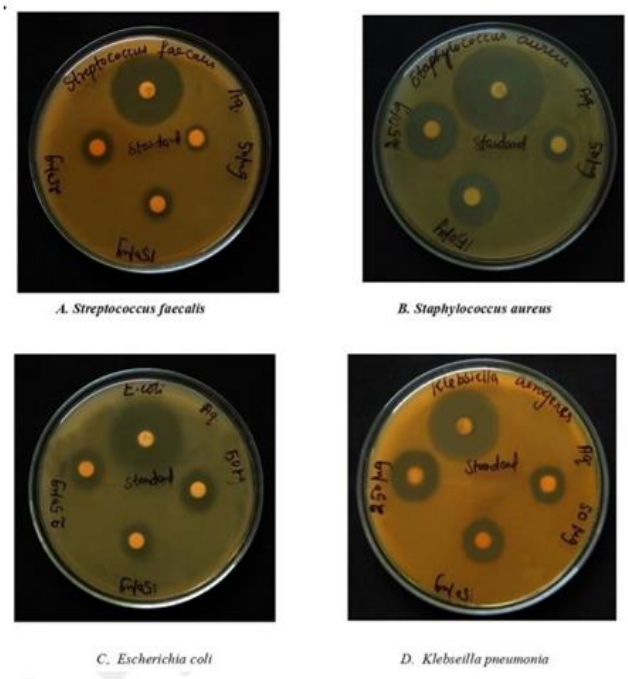

Fig. 5. Antibacterial screening of fucoidan against bacterial pathogens.

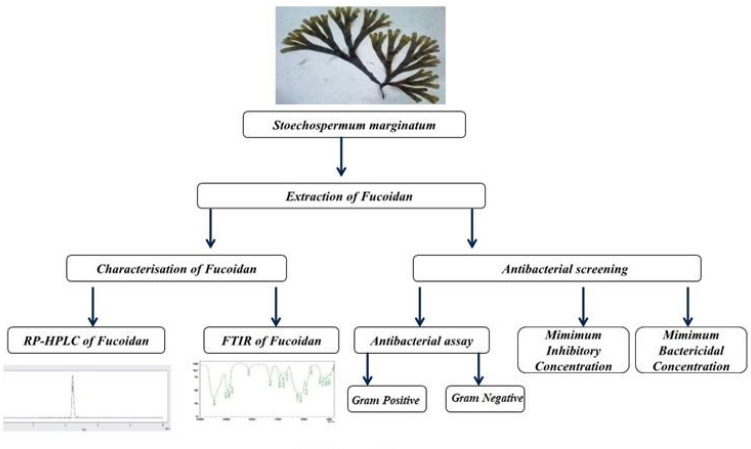

Fig. 6. Graphical abstract of the Isolation, characterization, and antimicrobial screening of fucoidan from brown seaweed (*S. marginatum*).

The brown seaweed *S. marginatum* collected along the coastline of Tamil Nadu was subjected to phytochemical and microbiological careening against clinical bacteria. The intracellular entrapped fucoidan was isolated by the acid hydrolysis method with a promising yield. The chemical structure of fucoidan was determined by chemical methods coupled with visible spectroscopy for the interpretation of fucose and total carbohydrates. The sulfate moiety was proved by the turbidity measurement method. The identity of the active principle fucoidan was confirmed after separation by conventional thin layer chromatography and modern RP-HPLC method which was evident when compared with the literature reference of fucoidan. Further FTIR spectroscopic analysis of isolated fucoidan has been proved by matching the functional group and fingerprint regions of the FTIR spectrum of the reference fucoidan with the isolated fucoidan from *S. marginatum*. The antimicrobial activity of fucoidan is related to its chemical structure and ester sulfate groups (Berteau *et al*., 2003). Researchers revealed that varied species of brown seaweeds with abundant sulfate content showed differences in antimicrobial activity against the different bacterial strains (Adhikari *et al.*, 2006, Asker, *et al.*, 2007).

The antibacterial efficacy of the isolated fucoidan when subjected to Minimum bactericidal concentration (MBC and Minimum Inhibitory concentration it delivered a noticeable antibacterial activity against clinical pathogenic bacteria of both gram positive and gram-negative strains. Fucoidan were found possess more antibacterial potency against gram-negative bacterial strains when compared with the gram-positive pathogens. The antibacterial activity against *K. pneumoniae* showed a MIC of 62.5 /mL and 150 /mL which is considerably higher when compared with the gram-positive bacterial strains.

CONCLUSION

Seaweed is found to be a rich and viable source of potent biomolecules. The present investigation on brown seaweed S. marginatum was subjected to acid extraction to isolate sulfated polysaccharides. The composition of fucoidan is further confirmed by the estimation of fucose and sulfate content. The isolated polysaccharides when analysed by TLC, HPLC and FTIR studies give solid evidence for the presence of fucoidan. Isolated fucoidan were screened for their antibacterial potential against gram-positive and gram-negative clinical pathogens. The antibacterial activity of fucoidan showed promising MIC and MIB. Fucoidan proven to be ideal lead molecules to cure various ailments; they can be utilized as a novel biomolecules for the cure of pathogenic bacterial diseases. Therefore, these results suggest that fucoidan will serve as herbal medicine with proven antimicrobial effects and may be useful for the treatment of pathogenic infections. The results obtained in the present study open up new possibilities for research on the use of this natural compound as a sustainable bioactive lead molecule. The future goal of the current study is to perform antimicrobial study on, pathogenic Virus, Fungus and protozoans, in addition to the entire bacterial pathogenic strains therefore, to eradicate clinical pathogens at the site of infection.

Ethical Approval. It is not applicable.

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Conflict of Interests. Authors have declared that no conflict of interests exists.

Author Contributions. Authors Balasubramanian Arul designed the antibacterial screening methodology and protocol, Saravanan Muniyappan performed the isolation, characterisation, and antibacterial screening. Author Kothai Ramalingam was involved in the proof correction and drafting of the manuscript.

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