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Antibacterial Properties of Fucoidan from Thirteen Indian Brown Seaweeds against Various Pathogenic Bacteria

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Authors' contributions

This work was carried out in collaboration among all authors. Author AMB is a Ph.D student who conducted the research and wrote the first draft. Authors LR, DLP and GC reviewed and corrected the first draft. Author SK is the supervisor of the Ph.D student who reviewed and corrected the final manuscript. All the authors read and approved the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The present study aimed to evaluate the antibacterial properties of hot water extracted fucoidan from thirteen Indian brown seaweeds against various pathogenic bacteria. The agar-well diffusion method was used to assess the antibacterial test of different fucoidan. The results revealed that *S. vulgare* fucoidan had higher antibacterial activity against many pathogenic bacteria, while fucoidan from other brown seaweeds had the least or no antibacterial activity in comparison to *S. vulgare* fucoidan. Therefore, we evaluated the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and brine shrimp toxicity assay in *S. vulgare* fucoidan. The MIC and MBC values were found to be 1.25 mg/mL and 10 mg/mL in *S. vulgare* fucoidan against pathogenic bacteria. *S. vulgare* fucoidan were found to be nontoxic as no mortality (Artemia) was found at different fucoidan concentrations. In addition, characterizations such as ultraviolet-visible spectroscopy, fourier-transform infrared spectroscopy, thermogravimetric analysis, differential scanning calorimetry, and scanning electron microscopy were studied in *Sargassum vulgare* fucoidan and revealed the properties of fucoidan. This study revealed that hot water extracted fucoidan may have other biological activities but not antibacterial action. Furthermore, studies may reveal the mode of action in fucoidan.

Keywords: antibacterial action; brine shrimp toxicity; MIC; pathogenic bacteria; structural characterization; sulfated polysaccharides.

1. INTRODUCTION

The emergence of antimicrobial resistance by many pathogenic bacteria has necessitated the search of many bioactives from various sources especially marine biologicals. Frequent use of antibiotics causes bacterial resistance to specific antimicrobial drugs and has a harmful influence on the host and environment. Researchers are focused on alternative sources of drugs to control pathogenic bacteria because most bacterial pathogens are becoming resistant to many antibiotics. Pathogenic bacteria can cause a wide range of diseases from mild to severe [1]. It has a significant impact on the socioeconomic status of individuals worldwide [2]. The bacterial genera causing infection are *Streptococcus* sp., *Staphylococcus* sp., *Escherichia* sp., *Pseudomonas* sp., *Aeromonas* sp., *Enterobacter* sp. and *Vibrio* sp. [3,4]. In general, antibiotics and chemicals are used to treat bacterial infections.

Marine macroalgae or seaweeds are a significant source of valuable macromolecules utilised in the nutraceutical and pharmaceutical sectors [5]. Sulfated polysaccharides (SPs) are a type of complex macromolecule that are found in seaweeds. Fucoidans, a complex sulfated polysaccharide that is a major component found in cell wall of brown seaweeds. It contains higher fucose and sulfate content with lower monosaccharide content [6]. The bioactivities of fucoidans from Indian brown seaweeds have been intensively studied by many authors, for example, antioxidant [7], anticoagulant [8], antimicrobial [9], anticancer [10] and immunomodulatory properties [11].

The antibacterial action of SPs derived from marine sources were widely reported. The acid extracted fucoidan from *S. polycystum* showed antibacterial activity against *V. harveyi*, *S. aureus* and *E. coli* [12]. The hot water extracted fucoidan from *S. swartzii* and *T. ornata* was sensitive to pathogenic bacteria [13,14,15-17]. Rani et al. [18] found that hot water extracted *P. tetrastromatica* and *T. ornata* fucoidan have stronger antibacterial activity against five aquatic pathogens. The unprocessed hot water extracted fucoidan from *L. japonica* could not show evident antibacterial action against *E. coli* or *S. aureus* [4]. The different SPs were sensitive to different microorganisms. However, the antibacterial mechanisms and structure-function links of SPs are to be elucidated.

The antibacterial activity of hot water extracted fucoidan from Indian brown seaweeds such as *S. asperum* [19] and *S. polycystum* [9] were reported. Here, we report the antibacterial action of hot water extracted fucoidan from thirteen Indian brown seaweeds. The objective of the present investigation is to evaluate the antibacterial action of hot water extracted fucoidan from thirteen Indian brown seaweeds using the agar-well diffusion assay. The best fucoidan that exhibit agar well diffusion method were further analysed by MIC, MBC, brine shrimp toxicity and structural characterizations

(ultraviolet-visible spectroscopy, fourier-transform infrared spectroscopy, thermogravimetric analysis, differential scanning calorimetry and scanning electron microscopy).

2. MATERIALS AND METHODS

2.1 Chemicals

The reagents and chemicals used acetone, calcium chloride, ethanol, Muller-Hinton agar, potassium bromide and TTC (2,3,5-triphenyl tetrazolium chloride) were purchased from HiMedia, India.

2.2 Sample Collection and Processing

Fresh Indian brown seaweeds viz., *Anthophycus longifolius*, *Colpomenia sinuosa*, *Dictyota dichotoma*, *Padina boergesenii*, *Padina boryana*, *Padina tetrastromatica*, *Sargassum cinctum*, *Sargassum prismaticum*, *Sargassum swartzii*, *Sargassum vulgare*, *Spatoglossum asperum*, *Stoechospermum polypodioides* and *Turbinaria ornata* were collected along the Gulf of Mannar region, South India during the period between August 2022 and January 2023. The brown seaweeds species were identified based on taxonomic literature [20-25] and cross verified [26-28]. The collected brown seaweeds were thoroughly cleaned with running tap water and then air-dried in the shade at 37°C. The dried seaweeds were then pulverized into powder using an electronic blender and stored in an airtight container.

2.3 Extraction of Fucoidan Using Hot Water

The hot water fucoidan extraction was carried out using the modified method [29]. To extract fucoidan, 100 g of different brown seaweed powder was added to 1 L of 85% ethanol and the mixture was kept in a magnetic stirrer for overnight. The solution was centrifuged at 3,000 rpm for 5 minutes and the supernatant was discarded. The collected residues were washed with acetone to remove the proteins. The leftover material was air dried on filter paper for 12 hours. The treated dried biomass (5 g) was added to 100 mL of distilled water (DW) and stirred at 65°C for 1 hour. The extraction process was repeated twice and the extracts were combined. The combined extracts were centrifuged at 12,000 rpm for 20 min and the supernatant was collected. The collected supernatant was combined with 1% $CaCl_2$ and kept in the freezer at 4°C for overnight and the mixture was centrifuged once again at 12,000 rpm for 20 min. The collected supernatant was made with 30% ethanol and incubated at 4 ºC. Then, the solution was centrifuged at 12,000 rpm for 20 min and the supernatant was collected. After that, the collected supernatant was made with 70% ethanol and the solution was kept at 4 ºC for the night. Then the solution was centrifuged at 12000 rpm for 20 min and the alcohol was decanted to collect the settled fucoidan. The settled fucoidan was washed with acetone and ethanol and the final product of fucoidan was dried at room temperature (RT). Then, the dried fucoidan was stored in an airtight container for further applications.

2.4 Bacterial Culture

In this study, pathogenic bacteria such as gram-negative bacteria (*Escherichia coli:* EC; *Klebsilla pneumonia:* KP, *Pseudomonas aeruginosa:* PA, *Enterobacter aerogenes:* EA, *Serraita marcescens:* SM, *Salmonella typhi:* ST, *Proteus mirabilis:* PM, *Vibrio* sp: VS) and gram positive bacteria (*Bacillus subtilis*: BS, *Staphylococcus aureus:* SA) were obtained from the Department of Microbiology, Manonmaniam Sundaranar (MS) University, Tirunelveli. A concentration of 10^7 CFU/mL was used.

2.5 Agar-Well Diffusion Assay

The antibacterial activity of fucoidan from thirteen brown seaweeds were assessed using the agar-well diffusion assay [30]. In petri plates, Muller-Hinton agar (MHA) medium was made with sterilised water. 0.1 mL of different selected bacterial strains were swabbed on agar medium with sterilised buds. Using a sterile well cutter, made 6 mm wells on the agar plates. Different fucoidan (10 mg/mL) was used as a sample and tetracycline (0.03 mg/mL) was utilised as a positive control. It was then pipetted into the appropriate wells using a sterile pipette. The plates were incubated for 24 hours at 37 °C. The antibacterial activity of fucoidan was determined by measuring the inhibition zone (mm) on the plates.

2.6 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of *S. vulgare* fucoidan were performed by the microdilution method using a 96-well plate [31]. The serial dilution of *S. vulgare* fucoidan was performed at different concentrations

between 10 mg/mL and 0.078 mg/mL. 100 µl of sterilised Mueller-Hinton broth was added to each well plate followed by 100 µl of *S. vulgare* fucoidan (different concentrations) added. After that, 10 µl inoculums of each bacteria were added to each well and cultured for 24 hours at room temperature. On each plate, a positive control (inoculums + no *S. vulgare* fucoidan sample) and a negative control (*S. vulgare* fucoidan sample + no inoculums) were included.

2.7 Determination of Minimum Bactericidal Concentration (MBC)

The MBC of *S. vulgare* fucoidan were evaluated by the standard protocol [31]. A loopful of *S. vulgare* fucoidan at different concentrations was streaked individually on petridish with MHA and incubated at 37°C for 18 hours. The MBC value for *S. vulgare* fucoidan is the lowest concentration of fucoidan that shows no bacterial growth.

2.8 Brine Shrimp Toxicity Assay

To investigate the toxicity of fucoidan, a brine shrimp (Artemia) toxicity assay was performed [13]. Brine shrimp (*Artemia salina*) eggs were hatched in a conical flask holding 500 mL of filtered saltwater for 48 hours. The conical flask was well aerated and incubated for 48 hours at 27°C with constant light. For this experiment, active nauplii were collected after hatching. *S. vulgare* fucoidan was dissolved in 1 mL of aerated saltwater at various concentrations (10 mg/mL, 50 mg/mL, 250 mg/mL, 500 mg/mL, and 1000 mg/mL). 1 mL of each concentration was added to 9 mL of aerated seawater. Each tube contained 10 nauplii. The control group contains 10 mL of seawater only. After 24 hours, the number of survivors was recorded and the death rate was determined as a percentage.

2.9 Structural Characterization

2.9.1 UV–visible spectroscopy (UV-VIS)

S. vulgare fucoidan (1 mg) was diluted in 1 mL of distilled water (DW) and the spectrum range was measured from 200 to 400 nm [32].

2.9.2 Fourier-transform infrared spectroscopY (FTIR)

The functional groups of the materials were analysed using an FTIR spectrophotometer. *S. vulgare* fucoidan (2 mg) was finely ground to a potassium bromide (KBr) grade of 1:10 using a spectra lab pelletizer. It was then inserted into the disc under vacuum. The infrared spectra were measured within the wave number range of 400 to 4000 cm^{-1} [18].

2.9.3 Scanning Electron Microscopy (SEM)

The surface morphology of *S. vulgare* fucoidan (2 mg) were observed using a scanning electron microscope. The morphological features were examined at various levels of magnification [33].

2.9.4 Thermogravimetric analysis and differential scanning calorimetry

The thermal characteristics of fucoidan were determined using DSC and TGA. *S. vulgare* fucoidan (15 mg) was employed in experimental circumstances at a rate of 5°C min^{-1} (with a range of 30-700°C) utilising a DSC/TGA instrument analyzer [34]. The reference utilised was an empty sealed aluminium pan and the nitrogen flow rate was set to 30 mL/min.

2.10 Statistical Analysis

The experiments were conducted in triplicate and the results were expressed as means ± standard error (SE).

3. RESULTS AND DISCUSSION

3.1 Agar Well Diffusion Assay

The antibacterial activity of different fucoidan was tested using the agar-well diffusion assay against many pathogenic bacteria (Table 1). *P. mirabilis* (20.66 ± 1.15 mm) in *S. vulgare* fucoidan and *S. typhi* (20.33 ± 1.52 mm) in *S. vulgare* fucoidan had the highest antibacterial activity. Whereas, *P. mirabilis* (5.66 ± 0.57 mm) in *S. polypodioides* fucoidan and *P. mirabilis* (5.66 ± 0.57 mm) in *P. boergesenii* fucoidan had the lowest antibacterial activity. Different fucoidan exhibited low or no antibacterial activity against all test microorganisms. Among different fucoidan, we evaluated MIC, MBC, BSTA, and structural characterization in *S. vulgare* fucoidan due to the presence of higher antibacterial activity in the agar-well diffusion assay.

In this study, fucoidan extracted using hot water from various brown seaweeds showed antibacterial activity against pathogenic bacteria such as *S. typhi* and *K. pneumoniae.* This finding contradicts the study of Chotigeat et al. [12], who

reported acid extracted *S. polycystum* fucoidan had highest antibacterial activity against pathogenic bacteria such as *V. harveyi*, *S. aureus* and *E. coli*. At a concentration of 500 mg/mL, Marudhupandi and Kumar [13] found that hot water extracted fucoidan from *S. swartzii* could kill *S. typhi* (8.6 ± 0.26 mm) and *Klebsiella* sp. (14.3 ± 0.41 mm). This result is dissimilar to the present study because hot water extracted *S. swartzii* fucoidan (10 mg/mL) inhibited the bacterial growth of *S. typhi* (18.66 ± 1.52 mm) and *K. pneumonia* (8.33 ± 0.57 mm). Yunhai [35] and Kordjazi et al. [36] reported small and clear inhibition zones around the paper disc on *S. aureus* and *B. subtilis* from hot water extracted fucoidan. However, no inhibition zones were observed against *S. aureus* and *B. subtilis* when using hot water extracted fucoidan from different brown seaweeds in the present study.

Rani et al. [18] observed higher antibacterial activity against five aquatic pathogens in hot water extracted *T. ornata* and *P. tetrastromatica* fucoidan, but no antibacterial activity was found in hot water extracted *S. marginatum* and *S. vulgare* fucoidan against many bacterial pathogens. According to Kantachumpoo and Chirapart [37], hot water extracted *C. sinuosa* fucoidan did not inhibit the microorganisms tested and this finding is similar to the present investigation. Even at 10 mg/mL, unprocessed hot water extracted fucoidan from *L. japonica* failed to exhibit significant antibacterial action against *E. coli* and *S. aureus* [4]. This finding is similar to the present investigation because different hot water extracted fucoidan exhibited no antibacterial activity.

Different fucoidan from other brown seaweeds had least or no antibacterial activity in comparison to *S. vulgare* fucoidan. This result caused may be due to the presence of higher carbohydrate content in the hot water extracted fucoidan. *S. vulgare* fucoidan exhibited better antibacterial activity may be due to the presence of higher sulfate content. The level of bioactivity is based on sulfate content in fucoidan. As shown in this study, hot water extracted fucoidan from different brown seaweeds had much smaller inhibitory zones against bacterial pathogens than the positive control. These findings suggested that bioactive molecules with antibacterial properties differed. Furthermore, it could be due to the concentration level that was utilized. Further investigation is required for fucoidan to fight various diseases.

3.2 MIC AND MBC

S. vulgare fucoidan had different concentrations ranging from 10 mg/mL to 0.078 mg/mL for MIC and MBC against different bacterial strains (Table 2). The MIC value of *S. vulgare* fucoidan, 1.25 mg/mL was shown to be the most effective against *S. typhi* and *P. mirabilis*. Whereas, 5 mg/mL showed the least effective against *K. pneumonia*, *P. aeruginosa*, and *Vibrio* sp. The MBC value of *S. vulgare* fucoidan, 10 mg/mL was shown to be the most effective against *K. pneumonia*, *P. aeruginosa*, *E. aerogenes* and *Vibrio* sp. Whereas, 5 mg/mL showed the least effective against *S. typhi* and *P. mirabilis.*

Many studies have shown that MIC and MBC of fucoidan from different bacterial pathogens were determined at various concentrations [9,12, 31, 38]. Different concentrations of *S. vulgare* fucoidan were determined in the present investigation and inhibited the visible growth of many pathogenic bacteria. In the present study, MIC and MBC values of different fucoidan were not always same. This might be because the fucoidan had different amounts of sulfate.

3.3 Brine Shrimp Toxicity Assay

The brine shrimp toxicity assay is regarded as an effective approach for conducting preliminary toxicity studies on a variety of bioactive compounds derived from different sources [39]. Parra et al. [40] observed that toxic nature of medicinal plants was strongly associated with brine shrimp lethality and oral lethal doses in mice. *S. vulgare* fucoidan at different concentrations was found to be nontoxic to brine shrimp (Artemia) in our study. This investigation supported the findings of [13]. No toxicity was found may be due to presence of different physiochemical characteristics present in hot water extracted fucoidan.

3.4 Structural Characterization

3.4.1 UV–Visible spectroscopic analysis

The UV-visible spectral analysis of *S. vulgare* fucoidan revealed a maximum absorbance peak around 260 nm (Fig. 1). This absorbance value confirmed as fucose-enriched sulphated polysaccharides [41]. Similar findings were observed for *Fucus evanescens* [42].

3.4.2 Fourier-transform infra-red analysis

The major polysaccharides are revealed by infrared spectra at 1200-800 cm^{-1} [43]. *S. vulgare*

fucoidan were recorded and shown in Fig. 2. The absorption bands at 2923.88 cm^{-1} and 2853.48 cm^{-1} in *S. vulgare* fucoidan show C-H stretching vibration. On the other hand, the absorption bands at 1638.82 cm^{-1} and 1400.5 cm^{-1} in *S. vulgare* fucoidan show the presence of a carbonyl group. In addition, wavelengths of 1033.31 cm^{-1}, and 869.92 cm^{-1} in *S. vulgare* fucoidan exhibit the presence of sulfate groups.

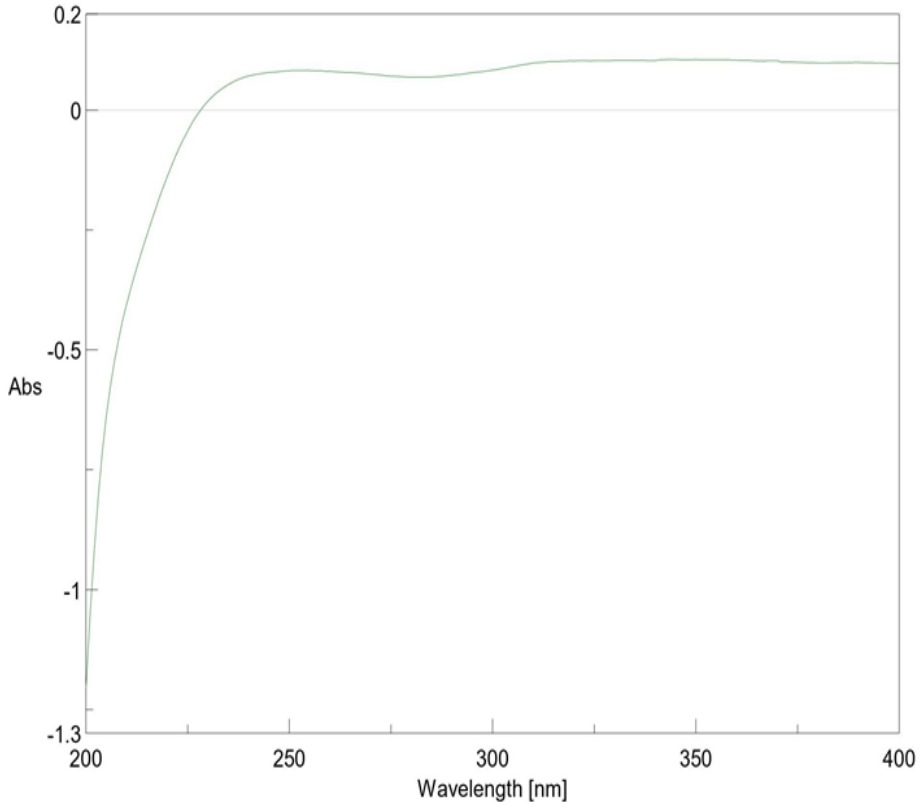

Fig. 1. UV-visible spectroscopy of hot water extracted *S. vulgare* fucoidan

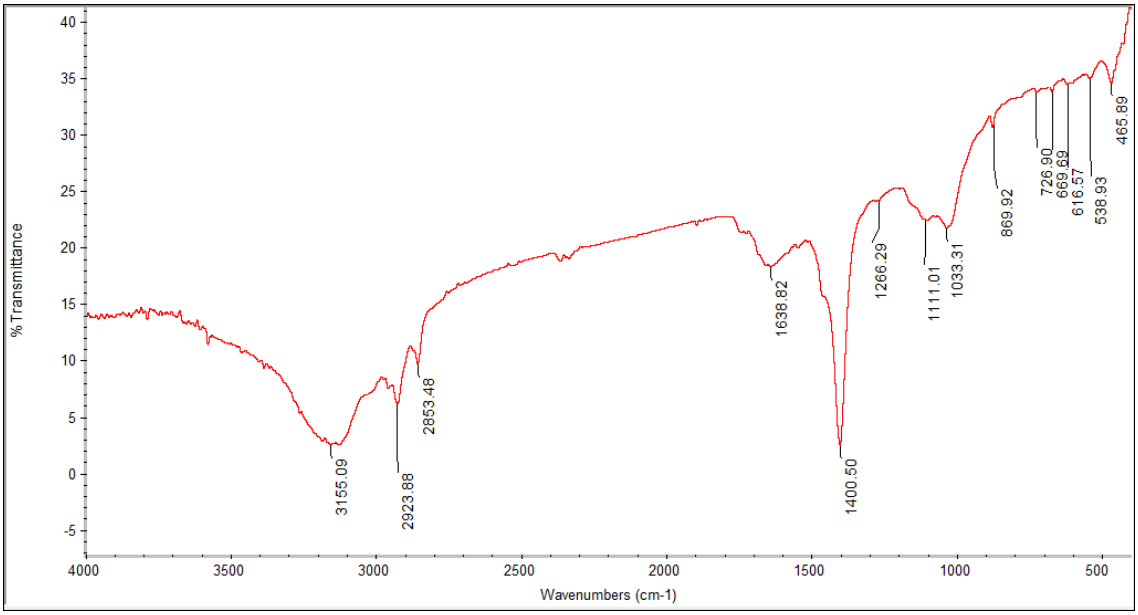

Fig. 2. FTIR spectroscopy of hot water extracted *S. vulgare* fucoidan

Table 1. Antibacterial activity of hot water extracted fucoidan using agar well diffusion assay from thirteen Indian brown seaweeds against pathogenic bacteria

	Diameter of inhibited zone (mm)									
Bacterial Species	**EC**	**KP**	**PA**	**EA**	**SM**	**ST**	**PM**	**VS**	**BS**	**SA**
S. prismaticum	0	9.66 ± 0.57	0	0	0	12.33 ± 0.57	0	0	0	0
A. longifolius	0	0	0	0	0	11 ± 1	0	0	0	0
S. cinctum	0	0	0	0	0	9.33 ± 0.57	0	0	0	0
S. vulgare	0	14 ± 1	11.66 ± 0.57	16.33 ± 0.57	0	20.33 ± 1.52	20.66 ± 1.15	16 ± 1	0	0
S. swartzii	0	8.33 ± 0.57	0	0	0	18.66 ± 1.52	0	0	0	0
T. ornata	0	0	11.66 ± 0.57	12.33 ± 0.57	0	6.66 ± 0.57	0	0	0	0
P. tetrastromatica	0	7.66 ± 0.57	0	0	0	0	0	0	0	0
P. boergesenii	0	8.33 ± 1.15	0	0	0	7.33 ± 0.57	5.66 ± 0.57	0	0	0
P. boryana	0	0	0	0	0	0	0	0	0	0
S. polypodioides	7.33 ± 0.57	0	0	0	0	6 ± 0	5.66 ± 0.57	0	0	0
S. asperum	0	7.33 ± 0.57	0	0	0	0	0	0	0	0
C. sinuosa	0	0	0	0	0	0	0	0	0	0
D. dichotoma	0	7.66 ± 0.57	0	0	0	0	0	0	0	0
Tetracycline	13.66 ± 0.57	11.66 ± 1.52	9.66 ± 0.57	14.66 ± 0.57	11.33 ± 1.52	20.66 ± 1.15	15.33 ± 0.57	18 ± 1	23.33 ± 1.52	11.33 ± 1.52

The experiments were carried out in triplicate and the data are represented as mean ± SE

**Escherichia coli: EC; Klebsilla pneumonia: KP, Pseudomonas aeruginosa: PA, Enterobacter aerogenes: EA, Serraita marcescens: SM, Salmonella typhi: ST, Proteus mirabilis: PM, Vibrio sp: VS) and gram positive bacteria (Bacillus subtilis: BS, Staphylococcus aureus: SA*

The absorption areas of 2930 cm^{-1} and 2853 cm^{-1} represent C-H stretching vibration [7,18]. During this investigation, these absorption zones were evident. The absorption zones between 1620 cm^{-1} and 1400 cm^{-1}, which have been observed in numerous investigations [7,38], showed the existence of a carbonyl group. These absorption zones have been identified in the present investigation. Absorption zones between 1030 cm^{-1} and 870 cm^{-1}, which were detected in various studies [44] indicated the presence of a sulfate group. As a result, *S. vulgare* fucoidan proved to be a sulfated polysaccharide.

3.4.3 SEM

SEM was utilized to assess the effect on the surface of fucoidan by examining morphological parameters [7]. Fig. 3 (a-c) shows SEM images of *S. vulgare* fucoidan at different magnification. It revealed a large surface area with overlapping/aggregation of oval-shaped particles, and the surfaces of the particles were rough. Sonia et al. [45] documented a SEM image of hot water extracted *S. swartzii* fucoidan and this results were similar to the present investigation except the study of Liu et al. [33] who captured a SEM image of *S. fusiforme* fucoidan extracted using HW, HA, and $Cacl_2$ methods. These variances could be due to differences in extraction conditions [38].

3.4.4 DSC and TGA

DSC is used to characterize heat-flow phase transitions in materials. DSC and TGA measurement curves of *S. vulgare* fucoidan are shown in Fig. 4. The results obtained clearly indicated the presence of endothermic peak around 130 ◦C respectively. TGA analysis presented three degradation phases to temperature ranges of 50°C, 100-100°C, and 150 °C. The first phase of heat degradation involves the loss of moisture from the samples. The major and minor devolatilization processes were defined by the second and third thermal degradation phases, respectively. The results reveal that *S. vulgare* fucoidan is thermally stable up to 150°C.

Table 2. Minimum inhibitory concentrations and minimum bactericidal concentrations of hot water extracted *S. vulgare* fucoidan against pathogenic bacteria

Bacterial pathogens	MIC (mg/mL)	MBC (mg/mL)
EC	0	0
KP	5	10
PA	5	10
EA	2.5	10
SM	0	0
ST	1.25	5
PM	1.25	5
VS	5	10
BS	0	0
SA	0	0

The study found a significant endothermic peak about 130 ◦C in hot water extracted *S. vulgare* fucoidan. Hanjabam et al. [46] found a single endothermic peak at 131.8 ◦C from hot water extracted *S. swartzii* fucoidan. The variation in heat flow might be explained by the material's chemical nature (sulphate content, monosaccharide composition, and polymerization degree) [47].

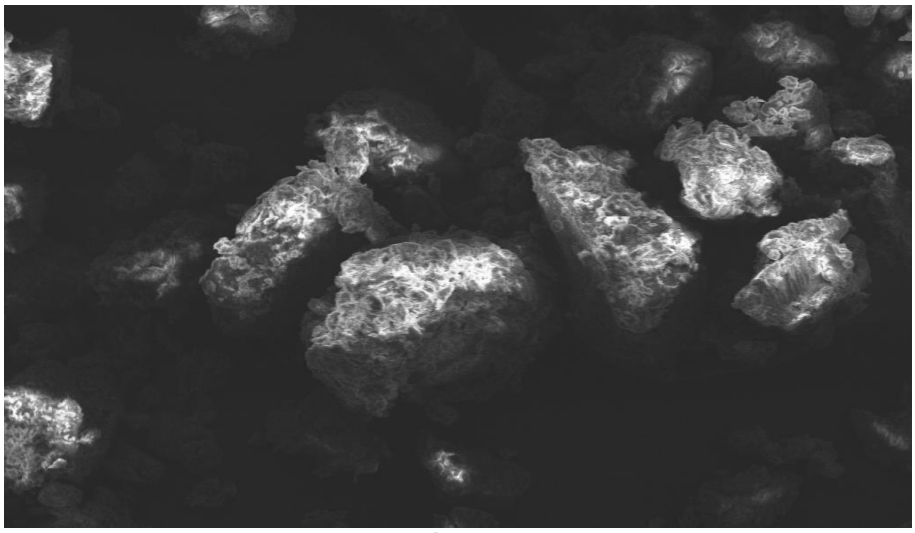

Fig. 3a

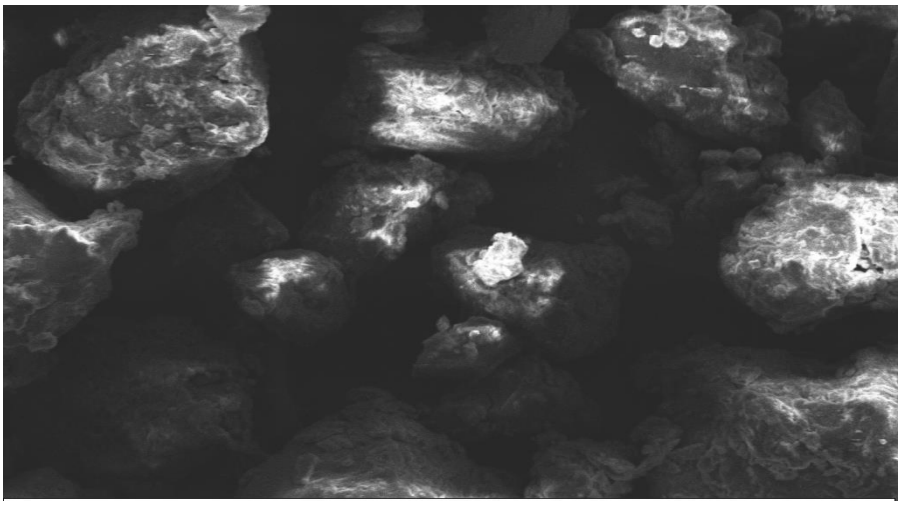

Fig. 3b

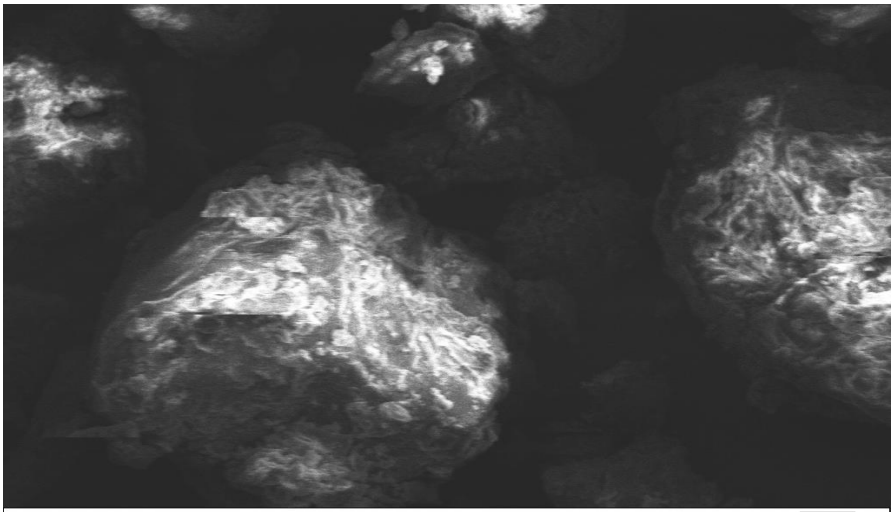

Fig. 3c

Fig. 3. SEM of hot water extracted *S. vulgare* fucoidan a) 1000x magnification b) 1500x magnification c) 2000x magnification

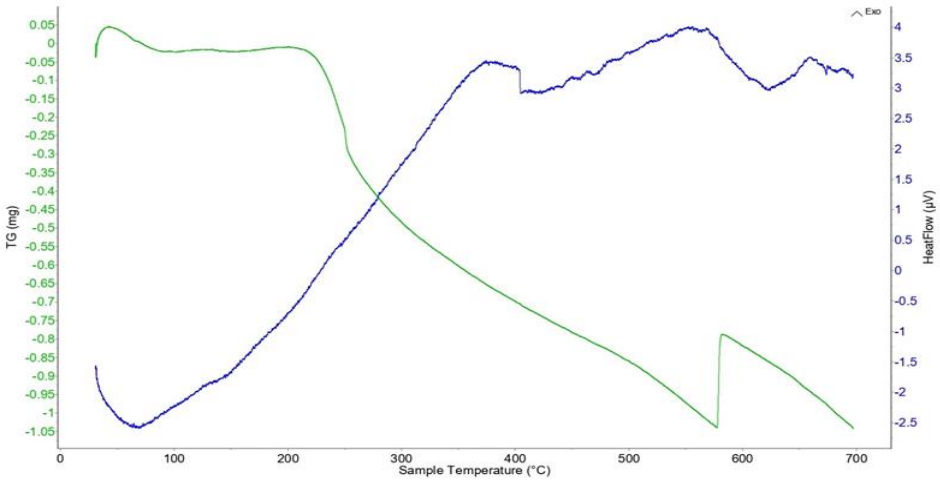

Fig. 4. DSC and TGA analysis of hot water extracted *S. vulgare* fucoidan

4. CONCLUSION

In the study, *S. vulgare* fucoidan were found to be more efficient against many tested pathogenic bacteria. While, fucoidan extracted from other brown seaweeds had little or no antibacterial effect against various pathogenic bacteria. The level of bioactivity in fucoidan is related to their chemical structure and ester sulfate groups. In addition, differences in antibacterial activity are also related to the marine environment in which the seaweeds grew. Further investigation into the mode of action of fucoidan is warranted based on the findings of this study.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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