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Isolation and Bioactive Potential of Fucoidan from Marine Macroalgae *Turbinaria conoides*

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The sulfated polysaccharide fucoidan was extracted from brown algae *Turbinaria conoides* using hot water and purified by anion-exchange chromatography. The biochemical and monosaccharide composition of purified fucoidan was studied by colorimetric assays and Gas Chromatography Mass Spectrometry (GCMS). The purity of fucoidan was confirmed by agarose gel electrophoresis and the structure was characterized by using spectroscopic techniques such as Fourier transform infrared spectroscopy (FT-IR) and solid-state nuclear magnetic resonance (NMR) techniques. Further, the bioactivity properties of fucoidan such as antioxidant, anticoagulant and anti-inflammatory were evaluated. The results showed profound antioxidant and anti-inflammatory activities of fucoidan. The anti-coagulant activity of fucoidan demonstrated striking inhibition of intrinsic and extrinsic coagulants involved in coagulation pathway.

Introduction

Brown seaweeds are the second most abundant group of marine macroalgae.[1] The richness of sulfated polysaccharides is one of the significant features of these seaweeds. Fucoidan is one type of complex sulfated polysaccharide found in the cell-wall matrix of the seaweed. In general, fucoidan contains mainly fucose, sulfate ester and uronic acid residues and other minor amount of monosugars. Most of the sulfated fucans described from fucales contain long stretches of alternating α-(1→3) and α-(1→4) L-fucose residues bearing one or two sulfate groups.[2] Biological activity of fucoidan is highly depends on its structure, molecular weight and chain conformations. Normally, fucoidan possesses complex, heterogeneous and indistinct building blocks such as sugars, sulfates and uronic acids with well-defined structural connectivity[3] and assigning exact structure is a challenging task. Significantly, these biomolecules provide a diverse range of biological properties like antiviral, anti-inflammatory and anticoagulant activities[4,5] make them to explore important area of research.

*Turbinaria conoides*is a marine algaecategorized in the class Phaeophyceae, order Fucales, family Sargassaceae, and genus *Turbinaria*. It has been found distributed worldwide in the sub-tropical and tropical regions. In contrast to other fucoidans isolated from various *brown algae*species, fucoidan from *Turbinaria* species is poorly investigated. Moreover, the fucoidan extracted from *T. conoides*is highly branched structure showed high antioxidant ability. The high branching nature of fucoidan extracted from *T. conoides*is still a challenge for assigning exact structure.[6]

In recent days, researchers rely on natural therapeutics for the well-being of society; hence, natural sources are being preferred for the treatment of various ailments. Fucoidan is a natural antioxidant and has the potential to eliminate free radicals.[7] Free radical induces cascade reactions that may cause deleterious effects in immune system. Therefore, it becomes necessary to include dietary supplements such as fucoidan to overcome all these effects.

Due to the ability to inhibit protein denaturation, non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used as pharmacological agents for the treatment of inflammation and pain. Numerous studies have been reported on the anti-inflammatory properties of seaweed extracts comprising polyphenols[8] and polysaccharides.[9] Since there are, only few evidences on the inhibition of protein denaturation by *Turbinaria*fucoidan is known, the present study focuses on the elucidation of various biological activities including anti-inflammatory activity.

Anticoagulant activity of sulfated polysaccharide has been extensively studied. Anticoagulant activity of fucoidan depends on molecular weight and length of the fucoidan chain[10] whereas the anticoagulant activity of other sulfated galactans depends on the nature of the sugar residue, the position of sulfate and total sulfate content in the polysaccharide molecule.[11] Thus, evaluation of anticoagulant property of *T. conoides*fucoidan is also a crucial aspect with respect to its structural conformations.

Thus, to address the anti-inflammatory and anticoagulant property of brown algae, *T. conoides*, the present study focuses

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/slct.201903548

on extraction of fucoidan from marine brown algae, *T. conoides*and most probable structural elucidation through FT-IR, Mass and solid-state NMR spectroscopic techniques. Furthermore, the pharmacological properties such as anti-inflammatory, antioxidant and anticoagulant activities have been evaluated.

Results and Discussion

Yield and Composition of Fucoidan Extracted fromT. conoides

The yield offucoidan is 3.2% from *T. conoides*on a dry weight basis by the hot water extraction method. Water extraction of fucoidan retains the natural bioactivity and maintains the stability of the molecule. The biochemical estimation of total fucose, sulfate and uronic acid content was found to be 54%, 37.98% and 27.96%, respectively. However, there was no protein content in the purified fucoidan. Significantly, the isolated fucoidan contains higher amount of sulfate (37.98%) and uronic acid (27.96%) compared to sulfate content (25.6%) and uronic content (7.8%) of other species *T. ornata*.[12] Thus, it should be noteworthy that the hot water extraction method yielded good quality of fucoidan with high content of sulfate and uronic acid. Therefore, it was feasible to confirm the higher purity and basic structural components of fucoidan.

FT-IR Characterization of Fucoidan

The FTIR results revealed the characteristic functional groups present in the isolated fucoidan (Figure 1). A strong peak observed at 3423 cm^{-1} indicates the presence of broad OH stretching, that in general, seen in all polysaccharides. A peak at 2929 cm^{-1} represents -CH stretching vibration. The peak centered at 1627 cm^{-1} showed the presence of carbonyl group (C═O group) from uronic acid.[13] The peak at 1254 cm^{-1} indicated the characteristic component of fucoidan, a sulfate group (S═O stretching) at C-4β position.[14] The peak at 1134 cm^{-1} indicated the stretching vibrations of the glycosidic C–O bond of fucoidan and other peak at 1055 cm^{-1} showed the stretching vibration of glycosidic bridge (C–O-C). The peak at 826 cm^{-1} indicates the equatorial sulfate groups at C_2 position. Thus, all the IR peaks observed were the characteristic signals of fucoidan as reported in the literature.[15]

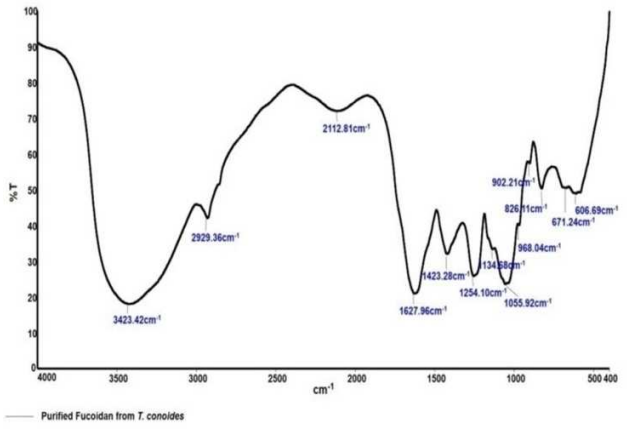

Figure 1. FT-IR spectrum of fucoidan obtained from *Turbinaria conoides*

Analysis of monosaccharide composition

The composition and number of sugar units present in the sample was estimated by GCMS analysis and compared with s retention time of reference standard sugars shown in Figures 2 and 3. As seen in the GCMS spectrum, fucoidan was found to have percentage of sugar units as follows: fucose (54.31%), galactose (24.6%), xylose (7.92%), glucose (3.75%), mannose (2.89%), rhamnose (1.95%), arabinose (2.05%) and ribose (2.5%).

Mass Spectrometric analysis of Fucoidan

The mass spectrum of hydrolyzed fucoidan of *T. conoides*is shown in Figure.3. The peak at *m/z* = 242.75 corresponds to the single unit of sulfated fucose residues like Fuc(4SO_3^-), Fuc (2SO_3^-), Fuc(3SO_3^-) as highest abundant base peak. The presence of galactose linkage with fucose at varied position namely, [Fuc_4 Gal (SO_3)]$^-$ was found from the peak at *m/z* = 843.60 (figure 3D), and from the figure 3C, the peak at *m/z* = 799.80 [Fuc_3 Gal (SO_3 Na)$_2^-$ - Na]$^-$, Gal(2SO_3^-)-(1,3)-Fuc-(1,3)-Fuc (SO_3^-)-(1,3)-Fuc(SO_3^-), Gal (2SO_3^-) -(1,3)-Fuc-(1,3)-Fuc (1,3)-Fuc

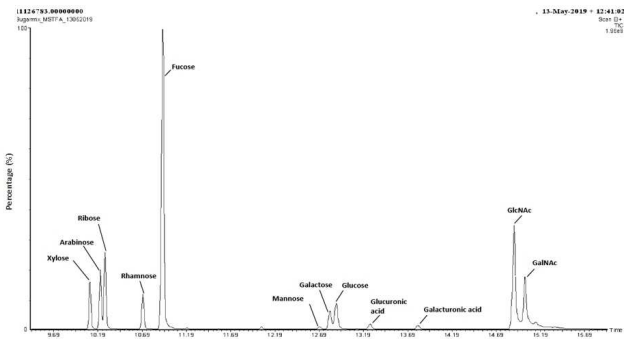

Figure 2. GCMS Chromatogram of sugars for purified fucoidan

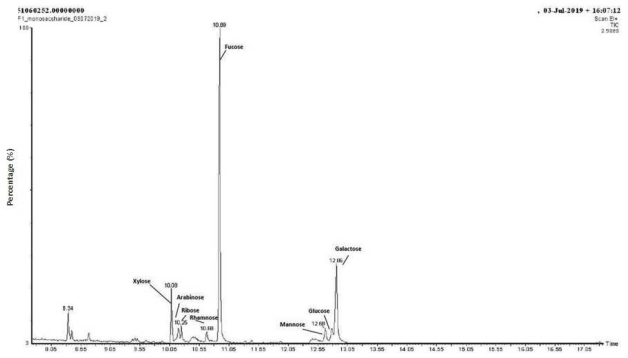

Figure 3. GCMS Chromatogram of sugar standard

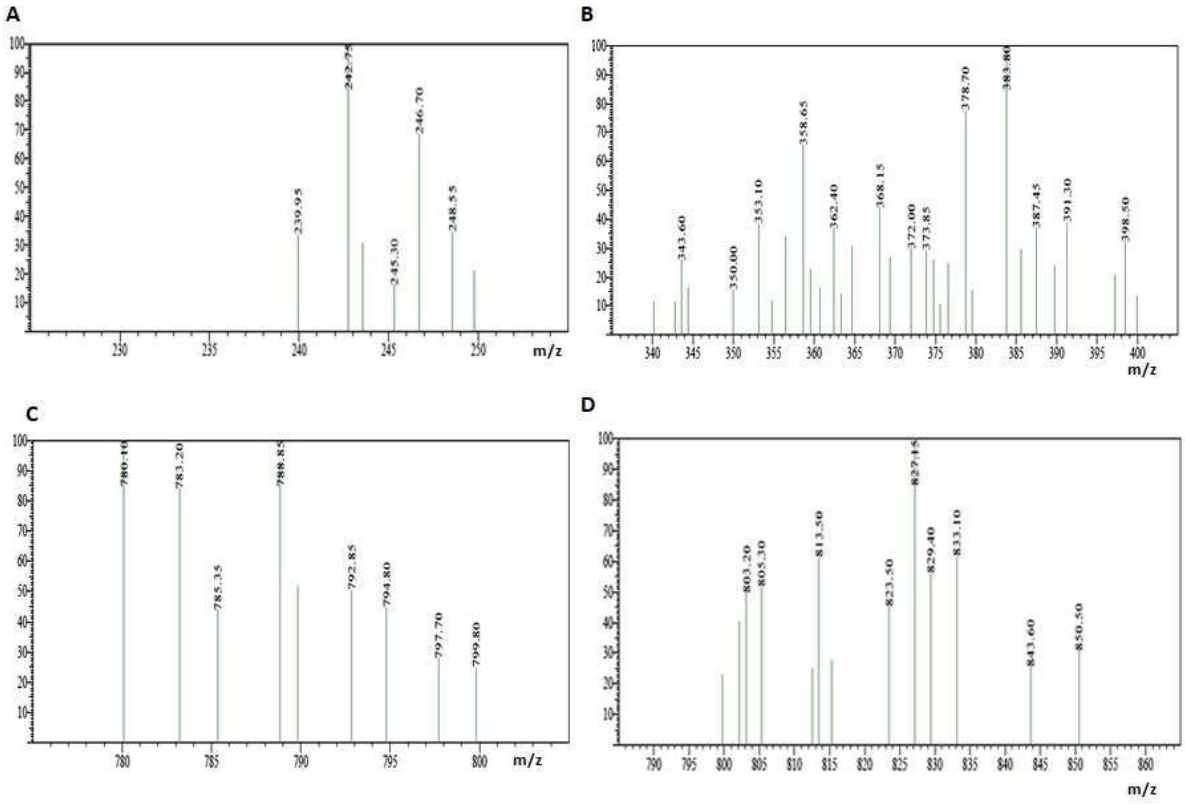

Figure 4. Mass spectrum (ESI-MS) of purified fucoidan extracted from brown seaweed *Turbinaria conoides*

$(2SO_3^-)$, Fuc-(1,4)-Gal$(3SO_3^-)$-(1,3)-Fuc$(2SO_3^-)$-(1,3)-Fuc$(2SO_3^-)$. A residual peak at $m/z = 373.85$ (figure 3B) was found due to fucose linked Xylose $[Fuc_1\ Xyl_1\ SO_3$ - Na$]^-$. Similar results were reported for fucoidan isolated from *Sargassum binderi*by Sinurat *et al.*, 2015 and *Coccophoralangs dorfii* by Anastyuk*et al.*, 2014. The MS spectrum from the figure 3B, 3 C, 3D represents large amounts of monosulfated fucooligosaccharides at $m/z = 242.75$, 827.15, 783.20, 343.60 and 387.45, respectively. Thus, the MS spectrum showed more relevance with the GCMS results and confirms the order of sugars units as Fucose > Galactose > Xylose.

Characterization of fucoidan by NMR Spectroscopy

The reports from earlier studies showed that the fucoidan from different algae possessed different structural configurations.[18] In order to record solution state ^{1}H NMR and ^{13}C NMR, the fucoidan sample was attempted to dissolve in D_2O and DMSO-d_6, however, it did not soluble in those solvents. Hence, the structural similarities of the purified fraction of fucoidan from *T. conoides*was investigated by solid-state ^{13}C cross polarization-magic angle spin (CP-MAS) method to study the presence of various types of carbon nucleus present in the sample (Figure 5). The spectrum exhibited intense chemical shift signals at δ 175.5 for the presence of carbonyl group, at an intense peak at δ 100.9 for the presence of CH carbon; peaks at δ 75.2 and δ 80.3 is significant and intense due to presence of large number

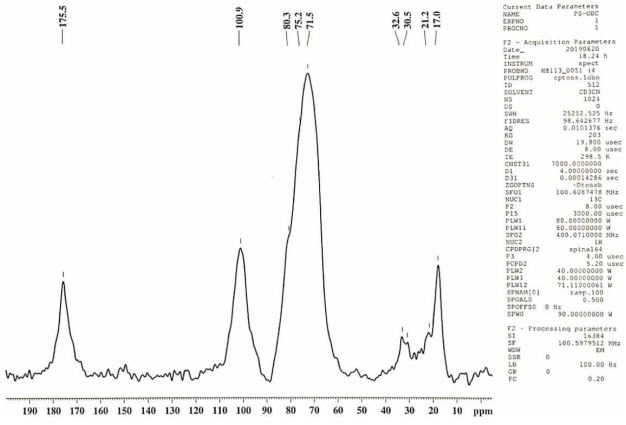

Figure 5. Solid state CP–MAS spectrum of fucoidan

of CH_2 carbons. Significantly, there are two types of OCH_3 carbons were observed at δ 17.0 and δ 21.2 due to α-orientation and δ 30.5 and δ 32.6 due to β-orientation of OCH_3 groups in the fucoidan.

The nature of primary, secondary and tertiary carbons present in the fucoidan sample was further confirmed by ^{13}C CPPI (Cross Polarization-Polarization Inversion) technique. Thus,the spectrum shown in figure 6 indicated a peak at δ 175.46 for carbonyl group and CH carbon at 100.9 gets cancelled, the CH_2 carbons at δ 71.50, 75.2, 80.3 gets inverted

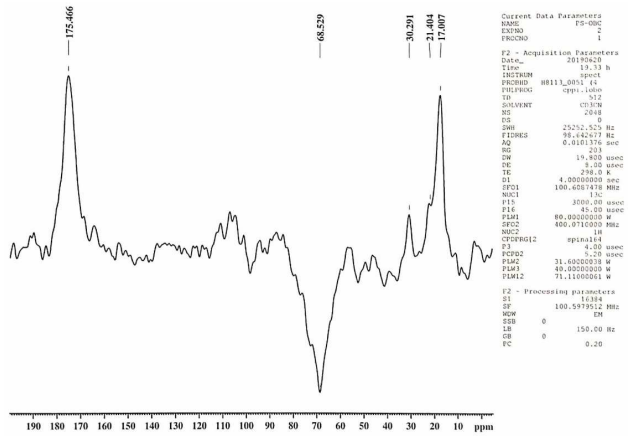

Figure 6. CPPI spectrum of fucoidan

to show negative peaks and peaks due to alpha and beta methoxy carbons appeared positive. The stacked spectra of CP-MAS and CPPI are shown is figure 7 for comparison. From the above study, it is evident that the fucoidan sample has significant carbons generally observed in fucoidan molecules and hence proposed basic structure is evident.

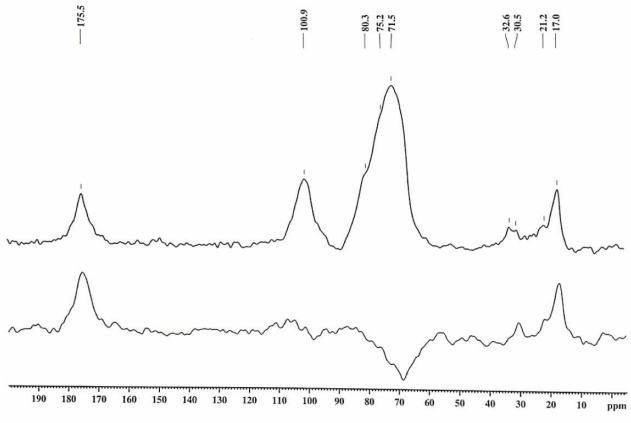

Figure 7. Staked spectra of CP–MAS AND CPPI

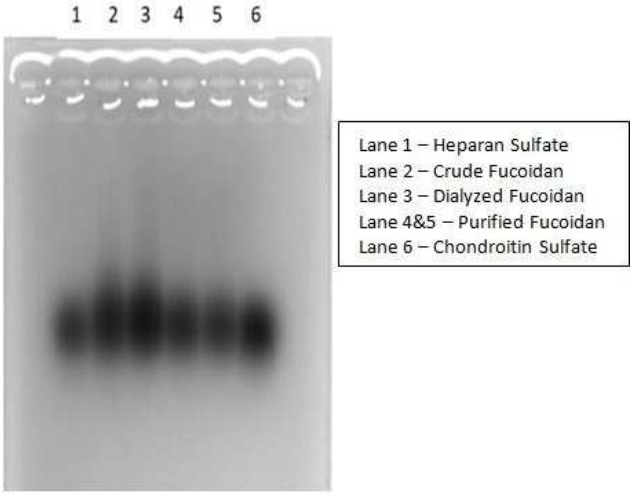

Figure 8. Gel Profile of Agarose Gel Electrophoresis

Agarose gel electrophoresis

The electrophoretic profile of agarose gel (Figure 8) confirmed the presence of fucoidan with a molecular weight of ~14 kDa with prominent bands and it was compared with the standards heparan sulfate 15 kDa (lane 1) and chondroitin sulfate 12 kDa (lane 6). The marginal variation in their electrophoretic mobilities was due to complex formed between sulfated polysaccharides and diamine that resulted in precipitation by CTAB.[19] These variations were due to the structure and sulfated patterns of the fucoidan.[20] Studies have reported that the presence of sulfate content in fucoidan is not affecting the electrophoretic mobility.[21] The banding patterns of crude (lane 2) and dialyzed fraction (lane 3) of fucoidan clearly showed the presence of minor impurity. Whereas the column-purified fucoidan (lanes 4 and 5) appeared as clear single spot after staining with toluidine indicated the purity of the sample unambiguously.

Biological activities of fucoidanAnti-inflammatory activity - Inhibition of albumin denaturation

Denaturation of proteins is one of the important causes for inflammation in conditions like rheumatoid arthritis, diabetes, cancer, etc.[22] Therefore, prevention of protein denaturation may help in reducing inflammatory responses. In the present study, *in vitro*anti - inflammatory activity of fucoidan showed a maximum inhibition of 93.28% compared to the reference drug, Diclofenac (96.6%) at the concentration of 100µg/ml. Gunathilake *et al.*, 2018 affirmed that the protection against protein denaturation is an important mechanism of action exhibited by NSAIDs. The *in vitro*anti-inflammatory study confirmed that the fucoidan possessed control of protein denaturation in a significant level.

Total Antioxidant activity

The total antioxidant capacity (TAC) was evaluated based on the reduction of phosphate molybdenum-VI to phosphate molybdenum-V by the purified fucoidan extract. The results indicate higher TAC of fucoidan extract (80.3%) at 100µg/ml. Further, it was observed that the total antioxidant activity was increased with increasing concentration of fucoidan (Figure 9). The free radical quenching activity of *T. conoides*fucoidan was almost equivalent to the standard, L-ascorbic acid exhibited 92.4% activity at 100µg/ml. Thus, the results indicated the antioxidant potency of fucoidan extract in free radical quenching is significant.

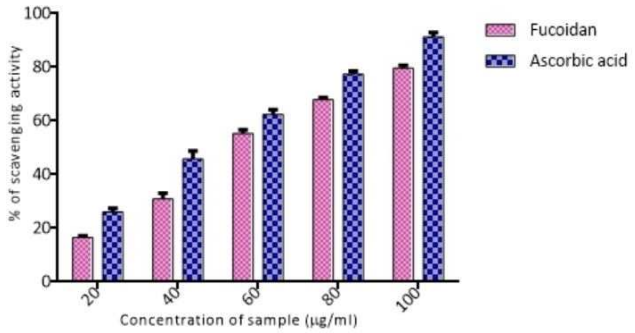

Figure 9. Total antioxidant activity of Fucoidan

DPPH radical scavenging assay

The DPPH possess proton free radical has characteristic UV absorption at 517 nm.[23] An excellent scavenging activity on DPPH radicals at a dosage of 100µg/ml (89%) was found for fucoidan compared to the ascorbic acid (95%) as control. Moreover, the conspicuous inhibitory effect of the fucoidan on DPPH radicals was found to be concentration dependent (Figure 10). The fucoidan extracted from *T. conoides* of 40 kDa showed only 61% of scavenging effect on DPPH radicals at a dosage of 5 mg/ml[6] whereas the fucoidan with 14 kDa in this study showed a better activity at low concentrations. Thus, the molecular weight of the compound has influence in their biological properties. The fucoidan at a concentration of 100µg/ml exhibited enhanced activity and thus, the same concentration was used for further biological analysis.

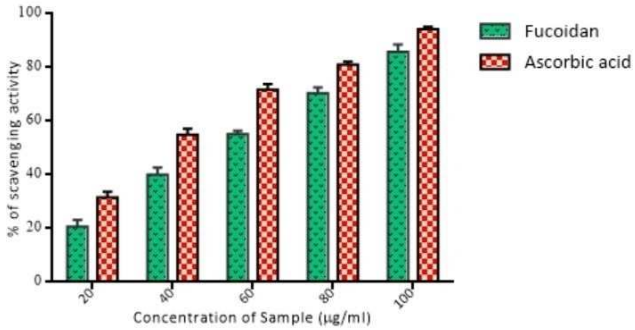

Figure 10. DPPH Free radical scavenging activity of Fucoidan

Table 1. Anticoagulant activity of fucoidan

Sample	Concentration(µg)	APTT(s)	PT (s)
Control (water)	–	30	–
Fucoidan	1	104	35
	5	244	73
	10	>300	128
Heparin	1	135	11
	5	>300	39

Anticoagulant activity

The anticoagulant activities of fucoidan were determined by activated APTT and PT assay. The results are shown in Table 1. The effect of fucoidan on clotting time was found to be different for APTT and PT. The prolongation of APTT by fucoidan recommended the inhibition of intrinsic factors such as VIII, IX, XI and XII in the coagulation pathway. The anticoagulant activity of the sulfated polysaccharides depends on their degree of substitution, molecular weight and the position of the sulfate group.[24] The effect of prolongation of PT activity suggested that the fucoidan inhibited the extrinsic pathway of coagulation. This activity also differs based on the quantity of uronic acid and conformational changes in the molecules of polysaccharides, fucoidan.[25] In this context, under *in vitro* condition, the prolongation of PT activity was found to be higher than the standard drug, heparin. Therefore, it could prolong the blood clotting time suggesting that fucoidan could be used as a therapeutic agent in surgical treatments

Conclusion

We have isolated sulfated polysaccharide fucoidan from *T. conoides* using hot water and it was found to be rich in fucose, galactose, and xylose with high sulfate and uronic acid content. The spectroscopic study such as FTIR and NMR confirmed that the fucoidan extracted from *T. conoides* was comprised of α–(1-2) – & α–(1–3) linked fucopyranosyl residues with two sulfate groups. Nevertheless, the repeating structure of this molecule was difficult to predict, as it is heterogeneous in nature and highly branched as per the results of mass spectrometry investigation. The antioxidantactivity of the fucoidan is attributed to their proton-donating activity that was evidenced through results of total antioxidant and DPPH radical scavenging activity. The results of the inhibition of protein denaturation by fucoidan from the *T. conoides*were the first of its kind as evidenced from this study. Based on the literature as well as the results, this study supports the fact that the presence of higher amount of sulfate content might be one of the prominent reasons for the potent biological activities of fucoidan. Furthermore, the purified fucoidan showed excellent anticoagulant activity equivalent to heparin as estimated through the APTT assay and it surpassed the clotting time of heparin in case of PT assay. The current study also reveals that owing to the pharmacological and medicinal properties, the *T. conoides*fucoidan could be used as a potential therapeutic agent for treating various ailments by strengthening the defense mechanism in the body.

Supporting information summary

Detailed information about the experimental procedures: Fucoidan extraction and biochemical analysis, sample preparation for Characterization, and experiments regarding the determination of biological potential of fucoidan are available in the Supporting Information.

Acknowledgement

The authors acknowledge MHRD for financial support. The authors thank CCAMP for GCMS analysis and CSIR-CLRI for solid-state NMR analysis. The authors also express gratitude to Dr. M. Arumugam, Associate Professor, DST sponsored National facility for Marine Natural Products and drug discovery research, CAS in Marine Biology, Annamalai University for Mass Spectrometry analysis.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Anticoagulant · Anti-inflammatory · Fucoidan · Seaweeds · Solid state NMR

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Submitted: September 20, 2019
Accepted: December 5, 2019