RESEARCH
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EXTRACTION AND PARTIAL CHARACTERIZATION OF ANTICOAGULANT SULPHATED CHITOSAN FROM PEN OF SEPIOTEUTHIS LESSONIANA

Running Title: Extraction and partial characterization of anticoagulant sulphated chitosan from pen of *SEPIOTEUTHIS LESSONIANA*

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Abstract

Introduction:
Sulfated chitosan is a modified form of chitosan, a naturally occurring biopolymer derived from chitin found in the exoskeletons of crustaceans and the pens of cephalopods.Chitosan itself has gained significant attention in various fields due to its biocompatibility, biodegradability, and versatile properties.The effectiveness of sulphation and the degree of substitution (DS) of sulphate groups will be assessed using partial characterisation techniques like elemental analysis and Fourier Transform Infrared Spectroscopy.

Materials and Method:
Chitin obtained from gladius was deacetylated by Takiguchi's technique and 40% aqueous NaOH to produce chitosan. 50 cc of DMF was used to prepare sulfated chitosan.In order to create gelatinous chitosan, SO3 was added to a 500 ml three-necked bottom flask holding 50 ml of chitosan solution in a solution of DMF and formic acid. After the reaction had been allowed to run for one to two and a half hours at the proper temperature of 40 to 60 °C, 300 cc of 95% ethanol were added to precipitate the product.Chitosan was extracted from the pen of the Sepioteuthis lessoniana using FT-IR, CHN, SEM, and XRD investigations, and it was then characterised. Purified chitosan was sulfated in N,N-dimethylformamide using chlorosulfonic acid, and the presence of the added sulfate group was confirmed via FT-IR analysis. Chitosan has high antioxidant properties, as evidenced by its capacity to scavenge DPPH, superoxide, and hydroxyl radicals as well as decrease and chelate ferrous ions.

Results:
When examined utilizing the activated partial thromboplastin time (APTT) and prothrombin time (PT), chitosan was discovered to be a powerful anticoagulant. According to the study's findings, S. lessoniana pen could be employed as an unusual source of natural anticoagulant which can be incorporated in functional food formulations.

Conclusion:
In the current study, the crystal clear structural reveal of sulfated chitosan synthesised from Sepioteuthis lessoniana was also clearly demonstrated, demonstrating the biological potential of the sulphate group as an efficient anti-coagulant.

Keywords: Sepioteuthis lessoniana; chitosan; FTIR; Anticoagulant, SEM analysis

INTRODUCTION*:*

Sulfated chitosan is a modified form of chitosan, a naturally occurring biopolymer derived from chitin found in the exoskeletons of crustaceans and the pens of cephalopods.(1) Chitosan itself has gained significant attention in various fields due to its biocompatibility, biodegradability, and versatile properties. However, by introducing sulphate groups onto chitosan, the resulting sulphated chitosan exhibits enhanced bioactivity, particularly in terms of its anticoagulant properties.(2,3)

Sepioteuthis lessoniana, commonly known as the bigfin reef squid, is a cephalopod species found in the Indo-Pacific region. The pens of Sepioteuthis lessoniana have been identified as a potential source of chitosan for various applications. (4) Extracting and characterising anticoagulant sulphated chitosan from the pen of Sepioteuthis lessoniana can provide valuable insights into the development of novel biomaterials with anticoagulant properties.(5)

Sulphated chitosan is a desirable choice for biomedical applications because of its anticoagulant qualities, such as the creation of antithrombotic coatings for medical devices, wound dressings, and drug delivery systems. (6) Sulfated chitosan is a possible substitute for traditional anticoagulant medications since it has the potential to limit the activity of blood clotting factors and prevent the formation of thrombi.(2)

In order to improve its anticoagulant capabilities, chitosan will be extracted from the pens of Sepioteuthis lessoniana and further modified by adding sulphate groups.(7) The effectiveness of sulphation and the degree of substitution (DS) of sulphate groups will be assessed using partial characterisation techniques like elemental analysis and Fourier Transform Infrared Spectroscopy (FTIR).(5,8)

The development of novel biomaterials with potential applications in the biomedical field, particularly in anticoagulation therapies and the prevention of thrombotic events, can benefit from an understanding of the extraction and partial characterization of anticoagulant sulphated chitosan from the pen of Sepioteuthis lessoniana(9)(10). The aim of the study is to extract and partial characterisation of anticoagulant sulphated chitosan from the pen of Sepioteuthis lessoniana.

MATERIALS AND METHODS:

The research was conducted in the Department Of Forensic Odontology , SAVEETHA INSTITUTE OF MEDICAL AND TECHNICAL SCIENCE. This research was done over a period of 3 months in the research department.

Extraction of chitin, chitosan and sulfated chitosan from pen of S.lessoniana

Takiguchi's method, which involved demineralizing and deproteinizing the S.lessoniana pen, was used to extract chitin. Chitin obtained from gladius was deacetylated by Takiguchi's technique and 40% aqueous NaOH to produce chitosan. 50 cc of DMF was used to prepare sulfated chitosan.In order to create gelatinous chitosan, SO3 was added to a 500 ml three-necked bottom flask holding 50 ml of chitosan solution in a solution of DMF and formic acid. After the reaction had been allowed to run for one to two and a half hours at the proper temperature of 40 to 60 °C, 300 cc of 95% ethanol were added to precipitate the product. It was filtered using a Buchner funnel under low pressure. The precipitate was washed with ethanol and then redissolved in distilled water. The pH was brought up to 7-8 using 2 M NaOH. The solution was dialyzed for 48 hours against distilled water using a dialysis membrane with a cutoff molecular weight of 12 kDa. The substance was concentrated and lyophilized to create sulfated chitosan.

FT-IR spectral analysis of chitin, chitosan and sulfated chitosan:

The FT-IR spectra of solid samples of chitin, chitosan, and sulfated chitosan collected from the pen of D. singhalensis as well as reference chitin and chitosan (Sigma) were obtained using the AVATAR 330 FT-IR spectrometer. In order to prepare the combination for additional spectrum analysis, the sample (10 g) was crushed into salt discs (10 mm in diameter) using 100 g of dried potassium bromide (KBr).

Scanning Electron Microscopy (SEM) of chitosan:

The surface morphology and microstructure of chitosan were investigated using a JEOL-JSM-5610LV with INCA EDS scanning electron microscope from Hitachi High-Technologies. Using direct evaporation at 20 volts, a thin layer of the gold/palladium (40/60) alloy was applied to the sample in the Hitachi Hus-4 vacuum evaporator. Various magnifications and an accelerated potential of 0.5 to 30 kv were used to test the preparation.

XRD Analysis of Sulphated Chitosan:

The partially defined chitosan can be submitted to X-ray diffraction examination following the extraction of sulphated chitosan from the pen of Sepioteuthis lessoniana.The chitosan sample's amorphous areas can be found using XRD. Lacking long-range organisation, amorphous patches can affect the material's characteristics.

Anticoagulant potential of sulfated chitosan:

The activated partial thromboplastin time (APTT) and prothrombin time (PT) kit were used to determine the anticoagulant potential of human plasma.

RESULTS:

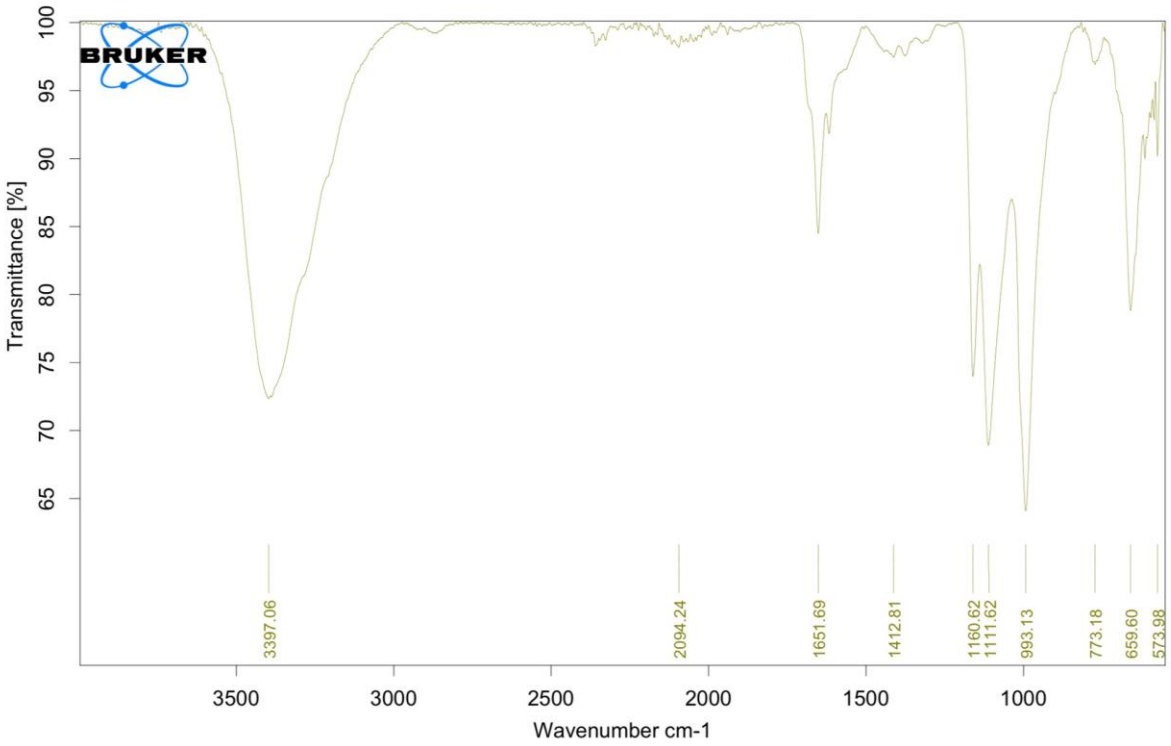

Fig:1 FTIR Graph for the absorption of hydroxyl ion and sulphated chitosan.

- The FTIR graph shows that the intensity of bands around 3370-3385 cm−1 in the IR spectrum was due to the hydroxyl stretching vibration of the sulphated chitosan and as expected they were broad.The absorptions at 1595-1610 cm−1 were assigned to the stretching vibrations of the CHO and C= O bonds.

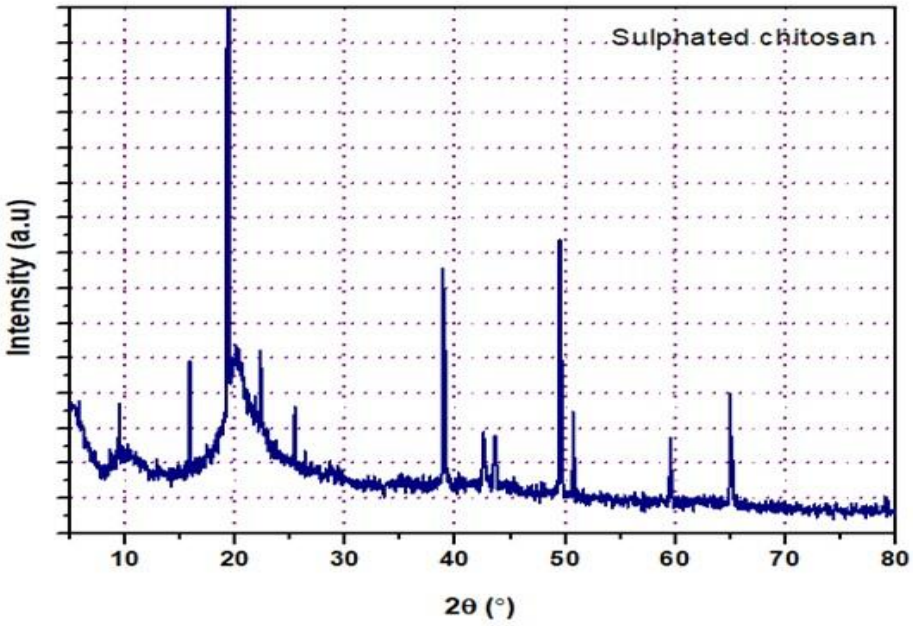

Fig: 2 XRD graph of polysaccharide i.e: chitosan.

- The polysaccharide displayed two weak peaks at around 2θ of 20°and 35°. However, the peak observed for chitosan at 2θ = 10°disappeared and the very broad peak at 2θ = 20°became weak in polysaccharide. The XRD pattern also indicated that the polysaccharide displays an amorphous form, which may participate in biomedical applications.

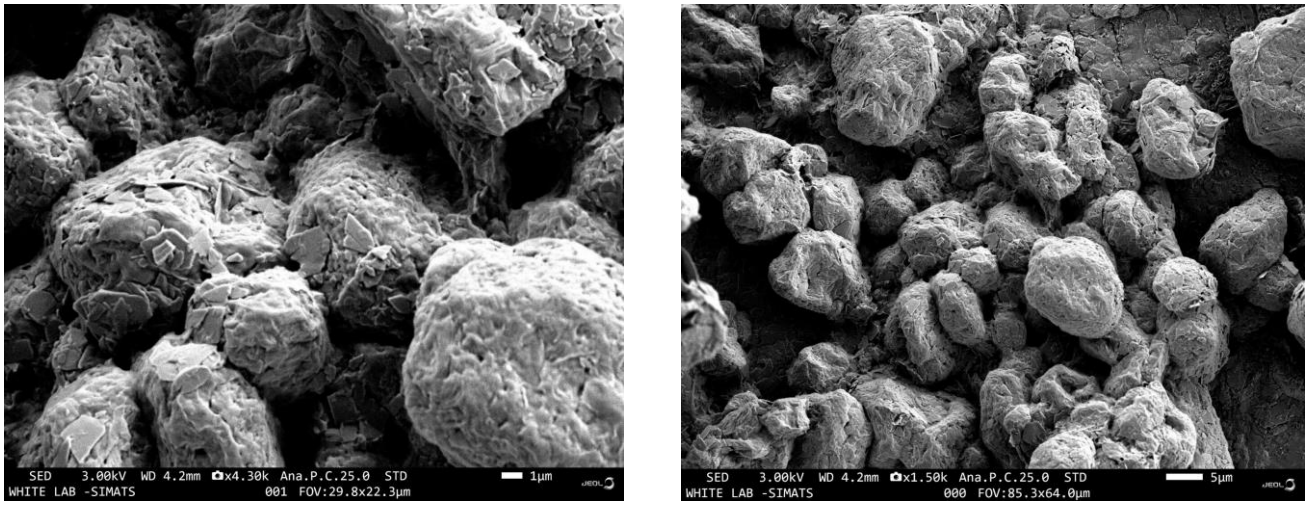

Fig: 3 SEM image of sulphated chitosan.

- The SEM image also confirmed the point that the sulphated chitosan has a near spherical morphology,which may participate in biomedical applications. It consists of a smooth membranous phase consisting of dome shaped orifices, microfibrils and crystallite.

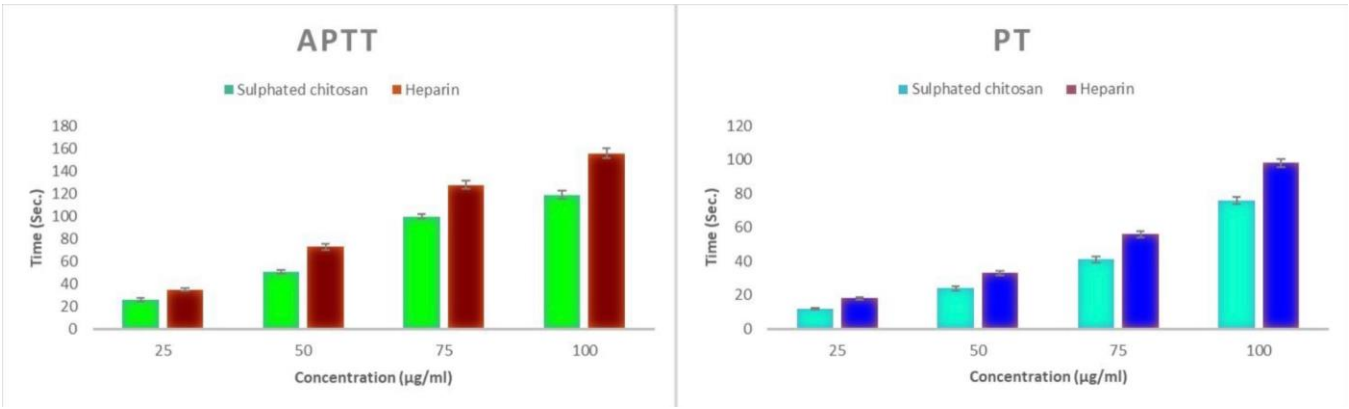

Fig: 4 The aPTT and PT graph which shows the anticoagulant property of suphated chitosan.

- The Activated thromboplastin time(aPTT) and Prothrombin time(PT) graph shows the anticoagulated activity of sulphated chitosan. The aPTT and PT graph depicts the time -concentration from 25-100 ul/ml.

DISCUSSION:

The skeleton structure of chitin has been shown to be remarkably similar to that of heparin, researchers have focused on using chitin for biological uses. The production of chitosan derivatives identical to heparin might be ensured by the deacetylation of chitosan, which is a critical process that occurs to varying degrees. It was achieved precisely by altering chitosan structurally at a constant condition. Researchers tested the sulfation reaction on chitosan, namely in a type of synthetic chitin heparinoid.(11)(12)

Even yet, the attempted sulfation results in one or more drawbacks, such as an insufficient conversion of the chitosan with a high degree of degradability. Because of its stability, intricacy, and availability, the sulfa-tion using N, N dimethylformamide (DMF) was shown to be more suited than previously reported methods. Additionally, polymers, polysaccharides, and polysaccharide derivatives were shown to be soluble in DMF.(11,13)

The basis for green FT-IR is that molecules' chemical bonds have inherent vibrational frequencies. The green infrared spectrophotometry stands out in this context since it is a technique that enables substance quantification without the use of organic solvents. Since medications with solubility issues can be examined in their solid form, it is appropriate for those drugs. Our present study shows that the intensity of bands around 3370-3385 cm−1 in the IR spectrum was due to the hydroxyl stretching vibration of the sulphated chitosan and as expected they were broad.

The absorptions at 1595-1610 cm−1 were assigned to the stretching vibrations of the CHO and C= O bonds.The bivalve Donax scortum's sulfated chitosan was reported to have absorption maxima at 668.90 cm-1 and 1134.36 cm-1, and the sulfo group was cited as the cause. The axial location of sulphate

in Ulva pertusa's spectrum was determined to be C-O-S, stretching vibration of C-O, S-O of sulphate, C-O of uronic acids, and O-H, respectively. These peaks were found to be at 847 cm-1, 1052 cm-1, 1641 cm-1, and 3446 cm-1.

Blood can coagulate under both in vivo and in vitro circumstances when anticoagulants like heparin and sulfated chitosan are used. Drozd et al. (2001) showed that the non-fractionated heparin derivatives of sulfated chitosan, in particular, cause the inactivation of thrombin and produce antithrombin III equimolar complex, also function as an additional anticoagulant.The study of anticoagulant sulphated chitosan from the pen of Sepioteuthis lessoniana provides a foundation for further investigations into its specific mechanisms of action, biocompatibility, and efficacy in various biomedical contexts.(14)

Despite having a larger anticoagulant capability than the sulfated chitosan synthesised from the gladius bone of Sepioteuthis lessioniana under semi-heterogeneous conditions in the current study, 51 ug/ml of APTT and 25 ug/ml of PT. Finding the effects of structurally modified sulfated chitins and sulfated chitosan on anticoagulant activity has been the main goal of this research. The idea that sulfated chitosan and its sulphate group position would favourably influence the APTT efficacy has also been substantiated by these results.(15)

CONCLUSION:

In the current study, the crystal clear structural reveal of sulfated chitosan synthesised from Sepioteuthis lessoniana was also clearly demonstrated, demonstrating the biological potential of the sulphate group as an efficient anti-coagulant. This work has demonstrated the use of sulfated chitosan as a powerful anticoagulant macromolecule. Sulfated chitosan from Sepioteuthis lessoniana gladius bone has powerful anticoagulant properties, making it a viable replacement for present anticoagulants. It might also be used as a food supplement to broaden the application of sulfated chitosan in the pharmaceutical and nutraceutical industries in the future.

LIMITATIONS:

Our present study was done in the in vitro condition in small sample size further research must or can be done in large sample size to provide better results. Much more assays need to be checked for the anticoagulative activity.

FUTURE SCOPE:

Our present study was done in invitro condition of extraction and partial characterization of sulphated chitosan from pen of S. lessoniana. Further research targeting animal models in vivo conditions that would substantially add anticoagulant properties and it would be a better drug of choice.

ETHICAL CLEARANCE:

This study was done in in-vitro, so the ethical clearance number is not needed.

CONFLICT OF INTEREST : There is no conflict of interest.

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AUTHOR CONTRIBUTION:

All authors are equally contributed.

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