RESEARCH
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SYNTHESIS OF CHITOSAN SULFATE FROM MARINE SQUID WASTE AND INHIBITION OF THROMBIN FORMATION IN HUMAN BLOOD

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Abstract
INTRODUCTION:
Loliolus investigatoris is a cephalopod(squid) belonging to the family Loliginidae. The pen or gladius of the squid is an internalized shell and serves as a protective barrier for the visceral organs. Gladius is often cut and discarded, but it consists of a very important homopolysaccharide, Chitin. Sulfated Chitosan is found to possess many biological properties like antibacterial, anti microbial,non toxicity and other properties.
MATERIALS AND METHOD:
Gladius of Loliolus investigatoris were taken and Chitin was extracted from the gladius. Chitosan was sulfated to form sulfated chitosan and was sent for characteristic analysis like FTIR, SEM and XRD analysis and Anticoagulant assessment tests like aPTT and PT.
RESULTS:
From the anticoagulant assays, we can infer that sulfated chitosan possesses similar and significant anticoagulant properties as compared to heparin which is a commercial anticoagulant used in surgeries.
CONCLUSION:
Chitosan sulfate prepared from the Gladius possess similar anticoagulant property to that of heparin and hence can be used as a potential anticoagulant in clinical and surgical uses.

KEYWORDS:
Loliolus investigatoris, squid, marine waste, anticoagulant activity, chitosan, chitin, chitosan sulfate, thrombin

INTRODUCTION:

Loliolus investigatoris is a cephalopod(squid) belonging to the Phylum- Mollusca, Class-Cephalopoda, Order-Teuthoidea, Family- Loliginidae and
Genus -Loliolus.

The pen or gladius of the squid is an internalized shell and serves as a protective barrier for the visceral organs. Gladius is often cut and discarded, but it consists of a very important homopolysaccharide, Chitin. Sulfated Chitosan is found to possess many biological properties like antibacterial, antimicrobial,non toxicity and other properties.

In recent years, the utilization of marine resources for various applications has gained significant attention due to its potential to address environmental concerns and contribute to the development of sustainable products. One such valuable marine resource is squid waste, a byproduct generated from seafood

processing industries. Squid waste contains chitin, a biopolymer that can be transformed into chitosan through deacetylation. Chitosan, a versatile biopolymer derived from chitin, has exhibited remarkable properties that make it attractive for a wide range of applications, including biomedical and pharmaceutical fields.

Chitosan is characterized by its biocompatibility, biodegradability, and non-toxic nature. These properties have led to extensive research focused on modifying chitosan to enhance its functional attributes. One such modification is the introduction of sulfate groups to chitosan, resulting in the formation of chitosan sulfate. This modification not only alters the physicochemical properties of chitosan but also imparts new bioactive properties that make it an intriguing candidate for various biomedical applications.

Thrombosis, the abnormal formation of blood clots within blood vessels, is a critical medical concern that can lead to serious health issues such as stroke, heart attack, and pulmonary embolism. Thrombin, a serine protease enzyme, plays a central role in the blood coagulation cascade and is a key mediator of thrombus formation. Inhibiting thrombin activity is a promising approach to prevent and manage thrombotic disorders. Conventional anticoagulant therapies often come with limitations such as bleeding risks and narrow therapeutic windows. Therefore, exploring alternative anticoagulant agents that effectively target thrombin while minimizing adverse effects is a significant area of research.

Chitosan sulfate, owing to its unique properties arising from the incorporation of sulfate groups, has shown potential as an anticoagulant agent. Studies have demonstrated that chitosan sulfate exhibits enhanced interactions with thrombin, leading to its inhibition. This interaction is thought to occur through electrostatic interactions between the negatively charged sulfate groups on chitosan sulfate and positively charged regions on the thrombin's surface.

This research aims to contribute to the synthesis of chitosan sulfate from marine squid waste and investigate its potential as an inhibitor of thrombin formation in human blood. By harnessing the bioactive properties of chitosan sulfate, this study seeks to develop a novel anticoagulant agent with improved specificity and reduced side effects compared to traditional therapies. The utilization of marine squid waste not only addresses sustainability concerns but also provides a cost-effective source of raw material for the production of chitosan sulfate.

MATERIALS AND METHODS:

This study was conducted in Saveetha Dental College for a span of 3 months. The study involved the extraction of gladius, a sea waste, from the squid species of interest- Loliolis investigatoris belonging to Phylum- Mollusca, Class-Cephalopoda, Order-Teuthoidea, Family- Loliginidae and
Genus -Loliolus.

Ethical clearance no:

Extraction of chitin and chitosan

Chitin was extracted from the gladius of Loliolis investigatoris by demineralization and deproteinization following the method described
by Takiguchi. Chitin, thus obtained from gladius was converted into chitosan by the process of deacetylation according to the method of Takiguchi using 40% aqueous NaOH.

Degree of deacetylation (DDA)

The degree of deacetylation (DDA) was determined by adopting the method of Lavertu et al. by using the stretching
patterns of 1H NMR spectrum following the equation DDA (%) = [H4.9/(H4.9 + H2.06/3)] × 100.

Other materials

β-Chitosan (Mw ∼ 246 kDa with deacetylation) from the gladius of squid S. lessoniana was used.

Butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylene diamine tetra acetic acid (EDTA), ferrozine and potassium ferricyanide were purchased from Sigma Chemical Co. (St. Louis, MO). Ferrous chloride and hydrogen peroxide were purchased from Merck Co. (Darmstadt, Germany). All other reagents used were of analytical grade.

Sulfation of β-chitosan:

Sulfation of β-chitosan was done following the method of Xing. 50 ml of DMF.SO3 was added into a 500 ml three-necked bottomed flask containing 50 ml of chitosan solution in a mixture of DMF–formic acid with swirling to get gelatinous chitosan. Then the reaction was run at adequate temperature (40–60 ◦C) for 1–2.5 h, and 95% of ethanol (300 ml) was added to precipitate the product. It was filtered through a Buchner funnel under reduced pressure. The precipitate was washed with ethanol, and then re-dissolved in distilled water. The pH was adjusted to 7–8 with 2 M NaOH. The solution was dialyzed against distilled water for 48 h using a 12,000 Da MW cut off dialysis membrane. The product was then concentrated and lyophilized to give sulfated chitosan.

Characteristic analysis of sulfated chitosan

The sulfated chitosan was sent for characteristic analysis such as FTIR(Fourier-transform infrared spectroscopy), SEM (Scanning electron microscope) analysis and XRD (X-Ray diffraction analysis).

In vitro anticoagulant activity

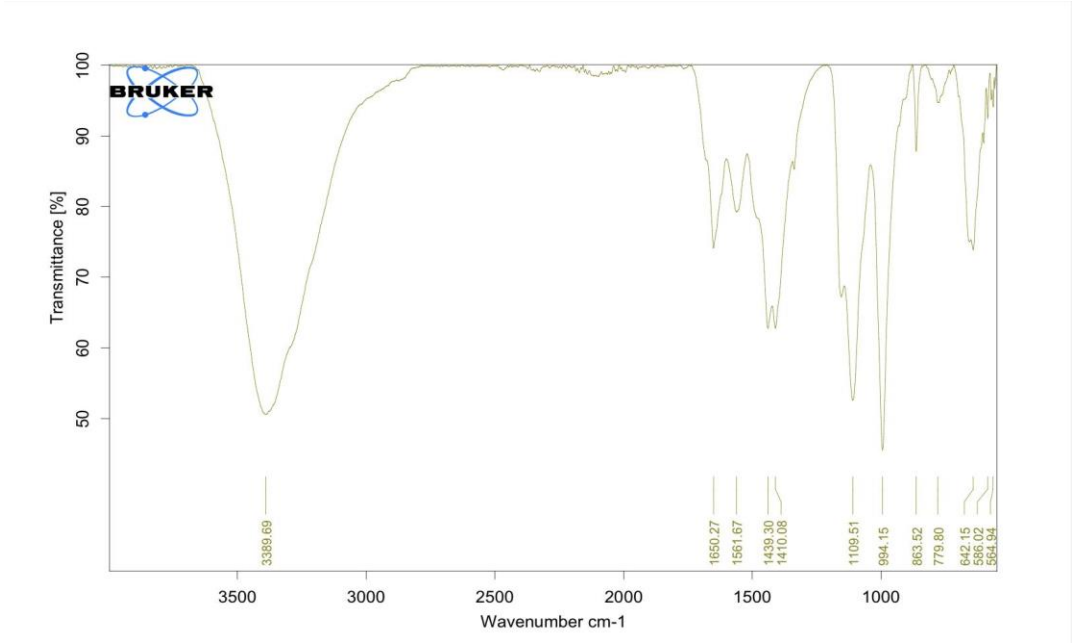
The anticoagulant activity of the sulfated chitosan was investigated by the classical coagulant assays, Activated partial thromboplastin time (APTT) and Prothrombin time (PT). APTT was performed using a kit obtained from Instrumentation Laboratory (Lexington, USA). The plasma (100 l) containing various concentrations of sulfated chitosan and heparin sulfate was incubated at 37 ◦C for 1 min. Bovine cephalin (100 l) was then added and incubated at 37 ◦C. After 3 min of incubation, 100 l of prewarmed 0.25 M CaCl2 solution was added to the mixture and the clotting time was measured and compared with the standard; The activity was expressed as IU. For determining PT (Lexing- ton, USA), 100 l of plasma containing various concentrations of sulfated chitosan was prewarmed for 5 min before adding 200 l of thromboplastin reagent. The clotting time was recorded simultaneously.

Statistical analysis

All data are expressed as means ± SD. Data were analyzed by analysis of variance ($P < 0.05$) and the means separated by Duncan's multiple range tests. The results were processed using MS-Excel and SPSS.

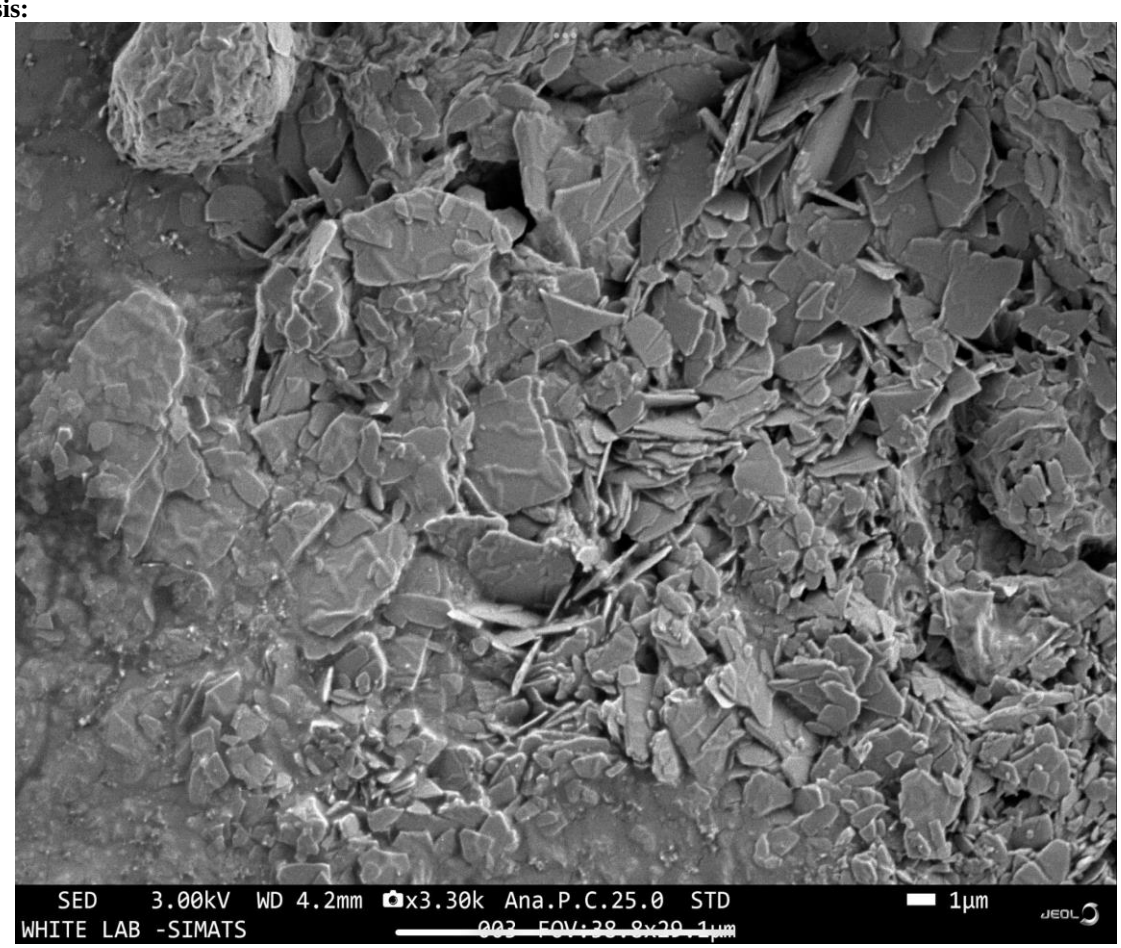
RESULTS:

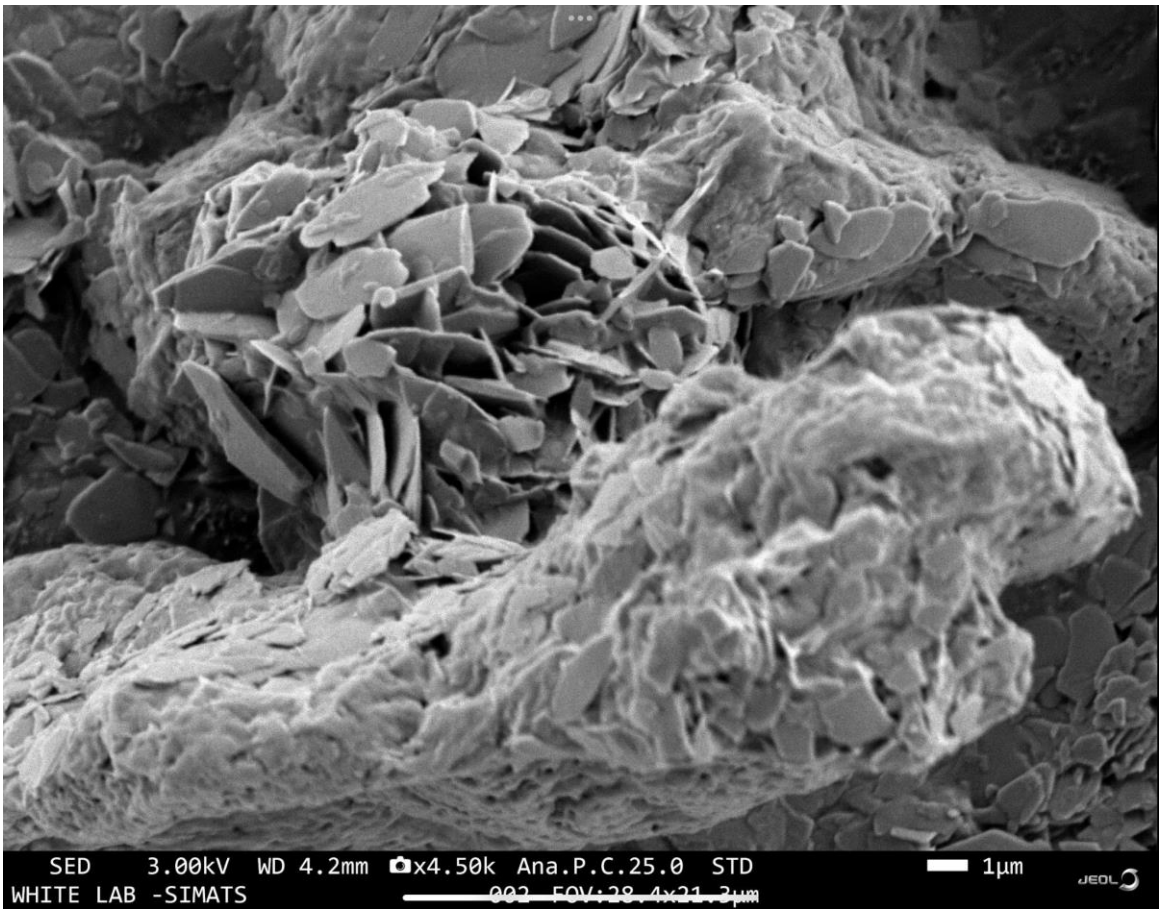
FTIR analysis:



The intensity band 3389.69 cm in the IR spectrum was due to the hydroxyl stretching vibration of the Chitosan sulfate and was expected to be broad. The absorptions at 1650.27 cm were assigned to the stretching vibrations of CHO and C=O bonds.

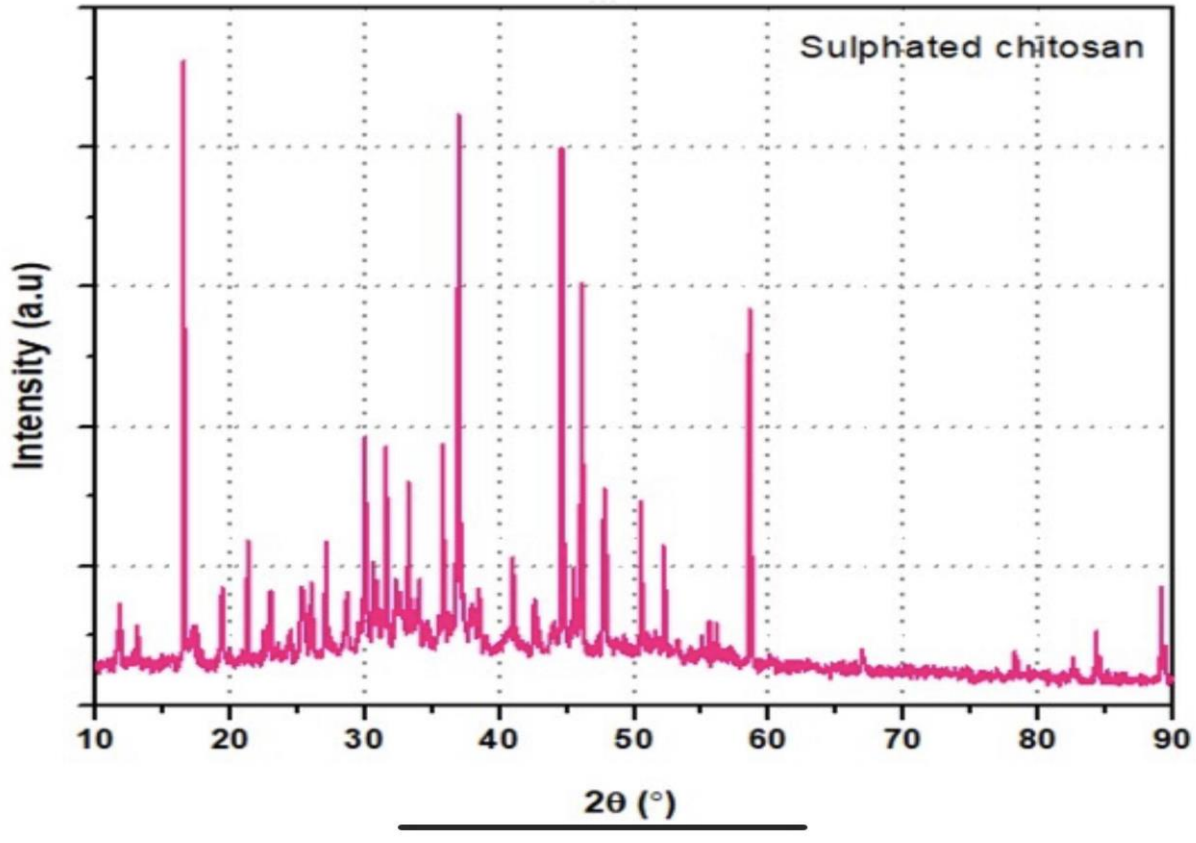
SEM analysis:





The SEM images of the Chitosan sulfate exhibited a nonporous, sheet-like and a rough surface. The SEM image also confirmed that the Chitosan sulfate has a sheet like morphology, which may possess many biomedical applications.

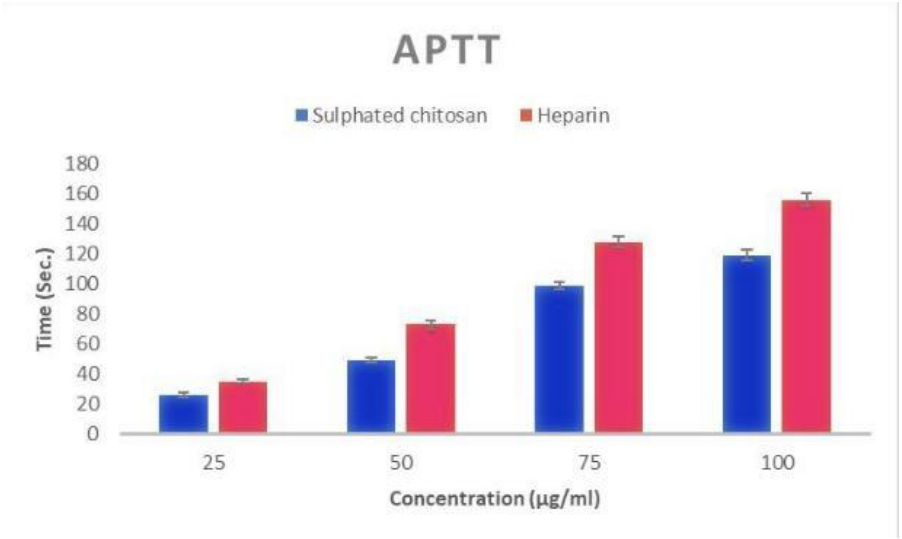
XRD analysis:



XRD studies of Chitosan Sulphate exhibit very broad peaks at 2θ=10° and 2θ=20°.
The Chitosan sulphate displayed weak peaks around 2θ of 30° and 40°. However the peak observed for Chitosan sulphate at 2θ=10° disappeared and the very broad peak at 2θ= 20° became weak in Chitosan sulphate.

Anticoagulant assay:

aPTT



PT assay

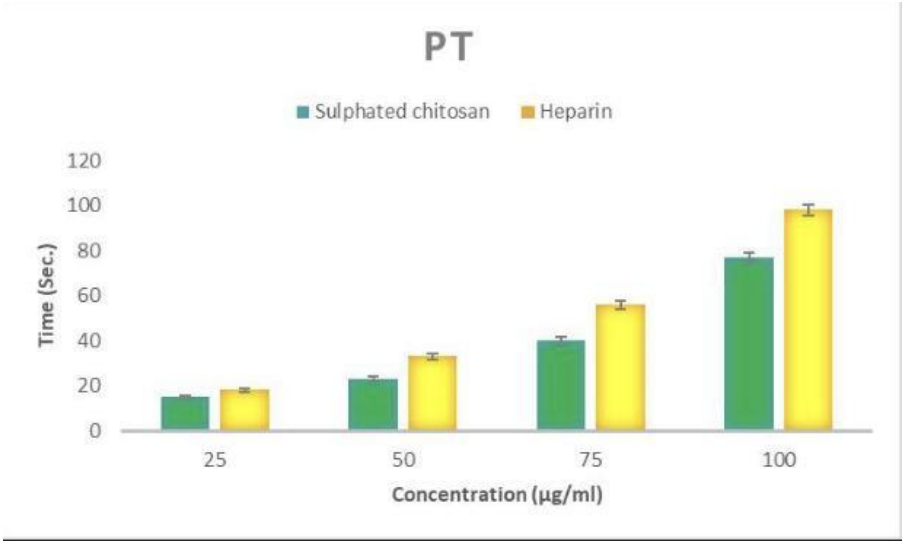


Table 1. Anticoagulant activity of Sulfated chitosan from *Loliolus investigatoris*

Concentration (µg/ml)	**Activated Partial Thromboplastin Time (APTT)**		**Prothrombin Time (PT)**	
	Sulphated chitosan (Sec.)	**Heparin (Sec.)**	**Sulphated chitosan (Sec.)**	**Heparin (Sec.)**
25	26±1.25	35±1.25	15±0.58	18±0.73
50	49±1.52	73±2.75	23±1.54	33±1.25
75	99±2.25	128±3.24	40±1.70	56±1.78
100	119±3.74	156±4.53	77±2.35	98±2.25

From the above table 1, we can infer that sulfated chitosan possess similar and high anticoagulant activity as compared to heparin, the commercial anticoagulant used in surgeries and in practice.

DISCUSSION:

From this study we can infer that Gladius of Loliolus investigatoris possess very significant anticoagulant properties. Some studies suggest that Gladius has other properties. Many molluscs like Sepioteuthis lessoniana have proved to possess anticoagulant properties and other properties like natural antioxidant properties and a potent food supplement source(Pasiyapphazham Ramasamy et al,2013). Chitosan from the cuttlebone of S. kobiensis could be used as another potential natural source of antioxidant,

possible food supplement or ingredient in the pharmaceutical industry(Annaian shanmugam et al,2013).

Other studies were also done on crustaceans to prove the anticoagulant properties of the extracted Chitosan sulfate (P.Ramasamy et al,2013).The findings of the above study is in accord with that of this study.

There are very limited resources on the anticoagulant activity of chitosan sulfate extracted from the waste products of marine molluscs. Hence this study can provide new literature on the anticoagulant activity of chitosan sulfate extracted from gladius of Loliolus investigatoris.

CONCLUSION:

The primary source of Chitosan is Prawn shells. This study provides an alternate source for Chitosan, which is the Gladius of Loliolus investigatoris, which is often removed and discarded. Chitosan sulfate prepared from the Gladius possess similar anticoagulant property to that of heparin, which is used commercially and hence can be used as a potential anticoagulant in clinical and surgical uses.

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CONFLICT OF INTEREST:

The authors hereby declare that there is no conflict of interest in this study.

REFERENCES:

1. *Y. Takiguchi, in: R.H. Chen, H.C. Chen (Eds.), Advances in Chitin Science, Proceeding from the third Asia-Pacific Chitin, Chitosan Jikken Manual, vol. 3, Gihodou Shupan Kabushki Kasisha, Japan, 1991, pp. 1–7 (Chapter 1).*
2. *Y. Takiguchi, in: A. Otakara, M. Yabuki (Eds.), Chitin, Chitosan – Jikken Manual, Gihodou Shupan Kabushki Kasisha, Japan, 1991, pp. 9–17 (Chapter 2).*
3. *3.M. Lavertu, Z. Xia, A.N. Serreqi, M. Berrada, A. Rodrigues, D. Wang, M.D. Buschmann, A. Gupta, J. Pharm. Biomed. Anal. 32 (2003) 1149–1158.*
4. *N. Subhapradha, P. Ramasamy, S. Vairamani, P. Madeswaran, A. Srinivasan, A. Shanmugam, Food Chemistry 141 (2013) 907–913.*
5. *R. Xing, S. Liu, H. Yu, Z. Guo, Z. Li, P. Li, Carbohydrate Polymers 61 (2005) 148–154*
6. *Ramasamy P, Subhapradha N, Srinivasan A, Shanmugam V, Krishnamoorthy J, Shanmugam A. In vitro evaluation of antimicrobial activity of methanolic extract from selected species of Cephalopods on clinical isolates. Afr. J. Microbiol. Res. 2011 Oct 23;5(23):3884-9.*
7. *Ramasamy P, Subhapradha N, Shanmugam V, Shanmugam A. Extraction, characterization and antioxidant property of chitosan from cuttlebone Sepia kobiensis (Hoyle 1885). International journal of biological macromolecules. 2014 Mar 1;64:202-12.*
8. *Sneka S, Preetha Santhakumar. Antibacterial Activity of Selenium Nanoparticles extracted from Capparis decidua against Escherichia coli and Lactobacillus Species. Research Journal of Pharmacy and Technology. 2021; 14(8):4452-4. doi: 10.52711/0974-360X.2021.00773*
9. *Vishaka S, Sridevi G, Selvaraj J. An in vitro analysis on the antioxidant and anti-diabetic properties of Kaempferia galanga rhizome using different solvent systems. J Adv Pharm Technol Res. 2022 Dec;13(Suppl 2):S505-S509. doi: 10.4103/japtr.japtr_189_22.*
10. *Sankar S. In silico design of a multi-epitope Chimera from Aedes aegypti salivary proteins OBP 22 and OBP 10: A promising candidate vaccine. J Vector Borne Dis. 2022 Oct-Dec;59(4):327-336. doi: 10.4103/0972-9062.353271.*
11. *Devi SK, Paramasivam A, Girija ASS, Priyadharsini JV. Decoding The Genetic Alterations In Cytochrome P450 Family 3 Genes And Its Association With HNSCC. Gulf J Oncolog. 2021 Sep;1(37):36-41.*