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Investigation of stingray spines by Fourier transform infrared spectroscopy analysis to recognize functional groups

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PEER REVIEW

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Comments

The venom of poisonous animals has been broadly studied because of their potential source as pharmacological activities. This is the valuable work on the sting ray spines towards the anti-venom synthesis. The study will be helpful for further pharmacological research to develop a treatment drugs or toxoids for poisonous animal injury and other purposes.

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ABSTRACT

Objective: To investigate functional groups of toxic spines in stingray by Fourier transform infrared spectroscopic analysis.

Methods: The venom extract of *Himantura gerrardi*, *Himantura imbricata* and *Pastinachus sephen* were centrifuged at 6000 r/min for 10 min. The supernatant was collected and preserved separately in methanol, ethanol, chloroform, acetone (1:2) and then soaked in the mentioned solvents for 48 h. Then extracts were filtered and used for Fourier transform infrared spectroscopic analysis.

Results: The results identified that the presence of free amino acids and protein having β -sheet and random coiled secondary structure. The presence of O-H stretch, C=O stretch, C-H stretch, N-H deformation, O-H deformation and C-O stretch in the sample aligned with standard bovine serum albumin. The influence of functional groups within the molecule was because of the impact of preferred spatial orientation, chemical and physical interaction on the molecule. In conclusion, compared to bovine serum albumin, *Himantura imbricata* consists of two C=O stretch, are involved in the hydrogen bonding that takes place between the different elements of secondary structure.

Conclusions: The venom of poisonous animals has been extensively studied, since standard medicine not available for treatment against injuries causing stingray. Therefore, it's the baseline study, to motivate further process and produce effective antibiotics.

KEYWORDS

FTIR, *Himantura gerrardi*, *Himantura imbricata*, *Pastinachus sephen*, Stingray spines, Polypeptides, Toxic spines

1. Introduction

Stingrays are cartilaginous fish that are grouped into four families: Gymnurid (butterfly rays), Urolophid (round stingray), Myliobatid (bat or eagle rays), and Dasyatid (proper sting rays)[1,2]. Rays fishes are typically encountered in the waters off of coastal regions and they are partially submerged into the sand[1-3]. When the ray is disturbed, it reflexively swings a barbed tail upwards, which can inflict deep puncture wounds[1]. The burbed tail has retro serrated

teeth making removal extremely difficult, which can lead to retained tail in the wound[2]. Most noticeably, stingrays have single or many formidable and arrow-shaped serrated spines at the base of the tail. They are generally used only as a defensive measure when caught, stepped on, or otherwise disturbed[2,4,5]. And these serrated spines are covered by an epithelia layer that has venom secretory cells and they are located in the epithelium or in close contact with it[6,7].

Serrated spines of rays may cause mechanical damage

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in victim's tissues and liberate venom to the injured tissues as well and there is no specific antidotal therapy for their venom[7]. In addition to producing traumatic injury, stingray tails have 1–4 stingers that release venom during an attack. Because the barbed tail is driven into the victim, a thin integument over the stinger ruptures, leading to envenomation[8]. Stingray injury in which the stinger penetrated the full–thickness of skin and embedded into the patient's bone. The injury resulted in a subcutaneous mass of granulomatous dermatitis and panniculitis with large zones of necrobiosis[9]. As a complicating factor, the sting might break and provoke the retention of dentine fragments in the wound. Bacterial infections especially that are caused by *Pseudomonas* sp. and *Staphylococcus* sp. are also commonly associated with these injuries[10,11].

Studies on toxicology and envenoming caused by elasmobranchs report mostly cases associated to stingrays of suborder Myliobatoidei[10], as they are the most clinically important since their venom may result in increasing local pain which may spread to involve the entire limb swelling and a characteristic bluish white appearance of the wound. The spines, including the venom gland, may be broken off in the attack and may remain in the wound which may be large and serrated and the patient experiences severe pain from the injected venom. Injuries made by ray's stings in some region of body such as thorax or abdomen can be accompanied by intense local pain and can cause moderate to severe complications such as nausea, vomiting, salivation, sweating, respiratory depression, muscle fasciculation's, convulsions, edema and ischemic necrosis[7,11–13].

Majority of injuries of stingrays have been reported from warmer tropical regions with their greater diversity of venomous marine creatures[3,10]. Meanwhile, stingray injuries to the trunk represent a special case requiring urgent hospitalization for investigation and management in case of bowel perforation, lacerated liver, and punctured lung or cardiac muscle[14]. Toxins from aquatic animals are an important strategy that guarantees their survival in a highly competitive ecosystem. These animals produce an enormous number of metabolic, whose combinations result in a great variety of chemical structures and complex molecules, such as alkaloids, steroids, peptides and proteins with chemical and pharmacological properties, different from that presented by the poisons of terrestrial animals[15]. There appear to be several different chemicals in the venom, but not all of these have been well characterized to date. Some authors describe neurotoxicity[15], cardio toxicity and circulatory disturbances[15]. Some studies demonstrated that venoms of rays contain serotonin, 5'-nucleotidase and phosphodiesterases[14].

Fourier transform infrared spectroscopy (FTIR) is a measurement technique whereby spectra are collected based on measurements of the coherence of a radioactive

source, using time–domain or space–domain measurements of the electromagnetic radiation or other type of radiation. Some of the major advantages of FTIR over the dispersive technique include speed, sensitivity, mechanical simplicity and internal calibration. FTIR provides identification of unknown materials, determination of quality of consistency of a sample and also the amount of components in a mixture. Studies on stingray from southeast coast of India are scanty. Therefore, the present investigation was carried out to identify the functional groups present in the crude extract of sting ray spines.

2. Materials and methods

2.1. Study area

Nagapattinam (Figure 1) coast has heterogeneous ecosystems like open sea, estuaries, mangroves, backwaters and industrial belt. Further, the areas have important fishing and landing center besides shipping harbors and a number of private industrial jetties with lot of fishing and industrial activities.

Nagapattinam (Latitude: 10°45.45 N; Longitude: 79°51.35 E) is one of the important fish landing centers of Tamilnadu. Cauvery river has alienated into number of branches, such as river of Vellaiaru, Kaduviar, Odampokkiaru and Vettaruare and finally they mixed to the Nagapattinam coastal waters[16]. Fishing activities are relatively moderate and both the conventional and non–conventional methods are adopted for effective fishing. Exports of high value of fishing products such as shrimp, crabs, lobster, cuttle fish, octopus, molluscs and edible fishes are exchange to foreign country. Majority of the ray fishes have been obtained from this station like stingrays, manta rays, devil rays[17].

2.2. Sample collection and preparation

Specimens [*Himantura gerrardi* (*H. gerrardi*), *Himantura imbricata* (*H. imbricata*) and *Pastinachus sephen* (*P. sephen*)] were collected with the help of local fisherman. Collected samples were raised with sterile water for removing of associated debris and salt. The epithelium (cover the sting) obtained from 60 animals (include three species) were scratched and grinded with phosphate buffer solution pH 7.4. The venom extracted was centrifuged at 6000 r/min for 10 min. The supernatant was collected and preserved separately in methanol, ethanol, chloroform, acetone (1:2) and brought to the laboratory. Samples were then soaked in the mentioned solvents for 48 h[18]. And they were filtered through Whatman No. 1 filtered paper. The filtrated crude samples were spined at 3000 r/min for 10 min. The supernatant was collected and used for further studies.

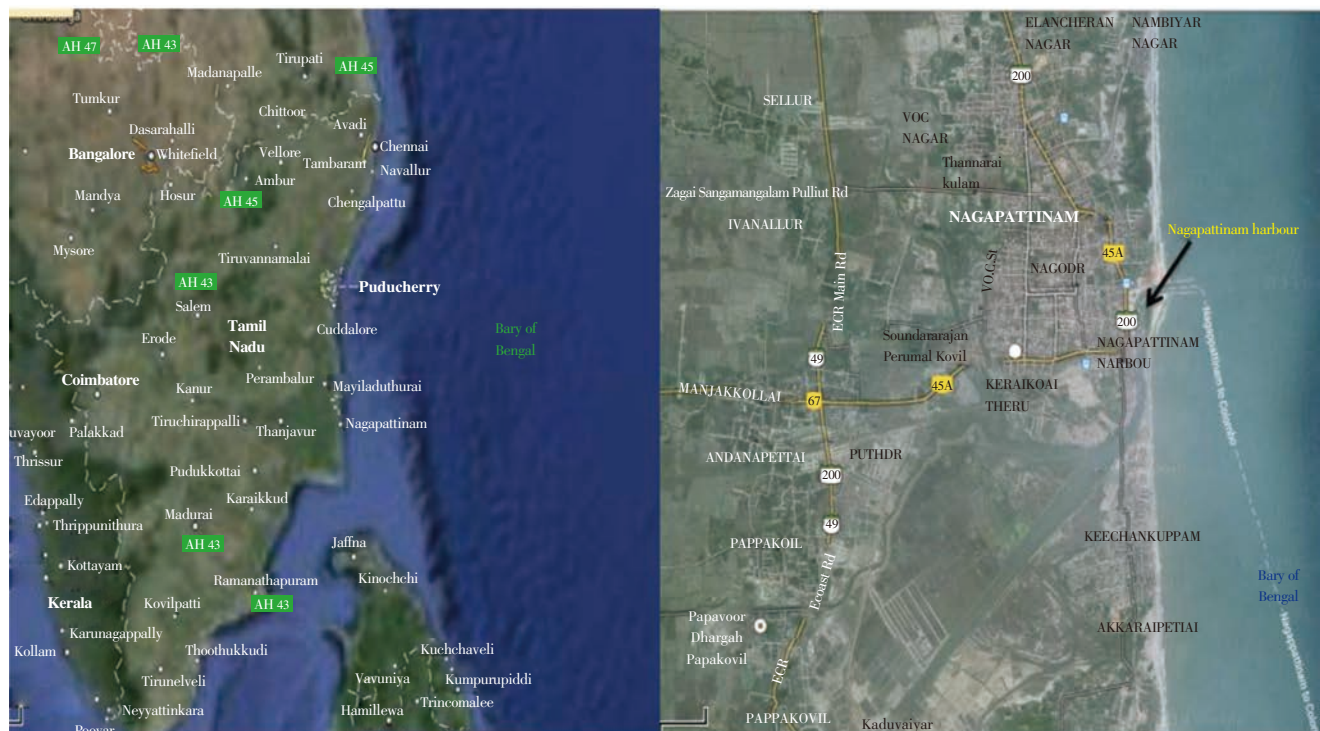


Figure 1. Nagapattinam landing center, Tamil Nadu, India.

2.3. FTIR analysis

FTIR spectroscopy of standard bovine serum albumin (BSA) and crude samples (*H. gerrardi*, *H. imbricata*, *P. sephen*) relied on IR affinity model Japan, (software name–IR solutions). About 10 mg samples were mixed with 100 mg of dried potassium bromide (KBr) and compressed further to prepare a salt disc (10 mm diameter) for reading the spectrum at Annamalai University.

3. Results

In the present study, the observations were exposed spines contain epithelial cells with distinct pigmentation (Figure 2) and arranged in the tail of the animal. Few rays have

one spine (*H. gerrardi*) while other groups have 1–4 spines. *H. imbricata* contains two spines, one overlap with another while placed at the dorsal part of the tail. The crude samples and BSA for standard were analyzed with FTIR spectroscopy. The results showed the variation of peak patterns of three samples, *H. gerrardi* (Figure 3), *H. imbricata* (Figure 4) and *P. sephen* (Figure 5) and with standard BSA (Figure 6) and it clearly showed all three samples of stingray spines had different bands patterns. General amide band pattern of the FTIR spectroscopy of control BSA and the samples were tabulated below (Table 1). The amide I band appears to exhibit at least five components, which were attributed to different secondary structure elements on literature assignments[9]. The frequency of component of the β -sheet vibrations, expected between 1640 and 1620 cm^{-1} , was not observed. The peaks between 1650 and 1660 cm^{-1} were generally assignment to α -helical absorption[19].

Table 1

General amide band patterning of the FTIR spectroscopy with stingrays.

Designation	Approximate Description frequency	Standard (BSA)	<i>H. gerrardi</i> venom	<i>H. imbricata</i> venom	<i>P. sephen</i> venom
Amid I	3 300–3 600 C=O stretch	3329	–	3337	–
Lipids	2 700–3 300 C–H stretch	2949	2927	2924	2929
–	2 700–3 300 C–H stretch	–	2366 2345	2365	2367
Amide I	1 600–1 690 C=O stretch	1654	1656	1660	1660
Amide II	1 500–1 700 N–H bending	1535	1559	1545	1549
	1 200–1 500 O–H bending (asymmetric NH_3^+ vibrations)	1450	1458	1454	1451
	1 200–1 500 O–H bending	1394	–	–	1340
	1 200–1 500 O–H bending	1296	1239	1240	1240
Free amino acids	900–1 300 C–O stretch	1097	1030	1027	1033
Free amino acids	800–880 Carbonates	927	876	876	873
Free amino acids	610–680 Sulphates	609	606	602	609
Amide VII	500–670 C–Br	534	561	561	561



Figure 2. Spines of different stingrays. A: Arrow showing location of spine in th dorsal part of the tail; B: Length of the spine collected from four different stingrays.

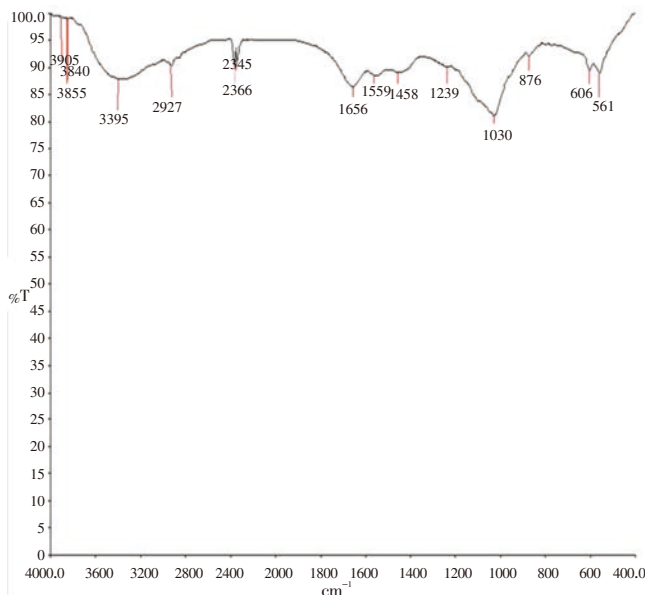


Figure 3. Graphically representation of sample 1 (*H. gerrardi*).

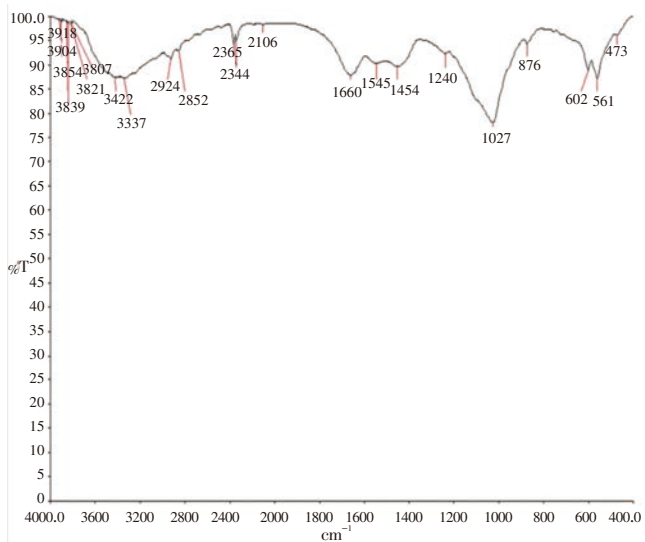


Figure 4. Graphically representation of sample 2 (*H. imbricata*).

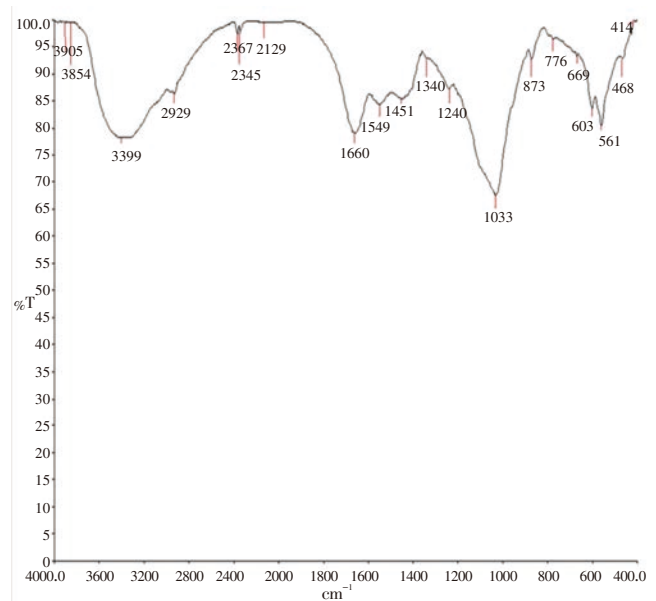


Figure 5. Graphically representation of sample 3 (*P. sephen*).

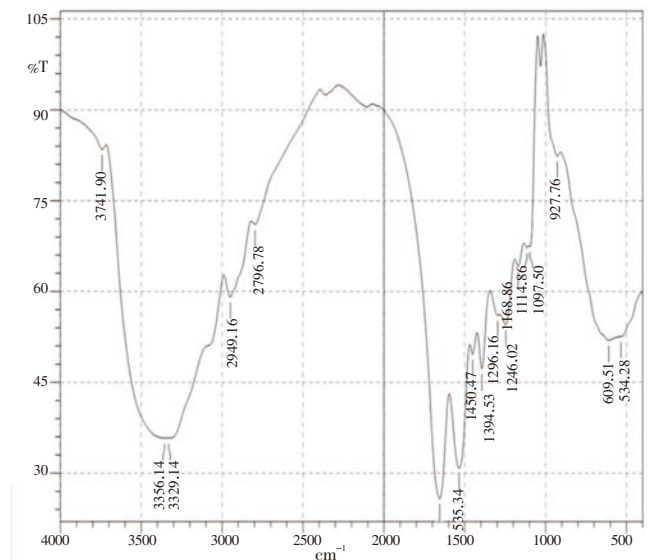


Figure 6. Graphically representation of BSA as a standard graph.

The α -helices peaks were presented at 3337 in *H. imbricata*, 1654 in BSA standard, 1656 in *H. gerrardi*, 1660 in *H. imbricata* and 1660 in *P. sephen* respectively in the sample. From the relative intensities of the amide I peaks, it would appear that the secondary structure of the protein. The amide II bands centered at 1535 in BSA standard, 1559 in *H. gerrardi*, 1545 in *H. imbricata*, 1549 in *P. sephen* indicate rapidly lose intensity or dissolution in D_2O due to H-D exchange. Peaks between 2700 and 3300 cm^{-1} were generally assignment to lipids absorption. Table 1 was obtained peaks at 2949 in BSA standard, 2927 in *H. gerrardi*, 2924 in *H. imbricata* and 2929 in *P. sephen* indicates C-H stretch present in the samples. Peaks between 900 and 1300 cm^{-1} were generally assignment to free amino acids absorption.

The peak demonstrated at 1097 in BSA standard, 1030 in *H. gerrardi*, 1027 in *H. imbricata* and 1033 in *P. sephen* indicates C-O stretch present in the samples (Table 1). Albeit peaks between 800 and 880 cm^{-1} are generally assignment to free amino acids absorption. Further, the carbonates results were obtained peaks at 927 in BSA

standard, 876 in *H. gerrardi*, 876 in *H. imbricata*, and 873 in *P. sephen* respectively. The free amino acids absorption peaks presented between 610 and 680 cm^{-1} are generally obligated in the sample. The sulfates were presented at the peaks of 1097 in BSA standard, 609 in *H. gerrardi*, 602 in *H. imbricata*, and 609 in *P. sephen* respectively. The peaks between 500 and 670 cm^{-1} are generally assignment to amide VII absorption. The C–Br result showed one peak at 534 in BSA standard, 561 in *H. gerrardi*, 561 in *H. imbricata* and 561 in *P. sephen* respectively in the sample.

4. Discussion

The venom of poisonous animals has been extensively studied because of their potential source as pharmacological agents and physiological tools. During the evolution, venomous animals developed highly specialized and sophisticated strategies that basically serve prey capture and/or defense purpose. The spines fixed in the fibrous tissue of the dorsal part of the root of the tail. The spine built from vasaodentine and covered with a layer of very hard vitrodentine. Laterally, on the ventral side, there were grooves that contain the glandular tissue, enveloped by the sheath^[2,4,5]. In the present study was observed the arrangement of spines, one overlap with another and the sheath patterns were showed by microscopic figure. In the course of the stinging act, the sheath breaks and the venom are mechanically expressed in the wound and glandular tissue is also found along the dorsum of the tail below the spine venom tissue if the satisfied epithelium in the ventral lateral grooves and the epithelium consist of about 4 layers of cells from the base to the surface^[20].

The venom contains phosphodiesterase, 5′-nucleotidase, and serotonin, and can cause both local and systemic effects. Locally, the venom triggers vasoconstriction and ischemia that leads to poor wound healing^[8]. The victim often reports intense pain^[2,21], out of proportion to the injury^[2,22]. In fact, the pain can be so severe that it leads to disorientation in the victim^[6]. Systemically, the venom can cause weakness, diaphoresis, nausea, vomiting, diarrhea, dysrhythmias, syncope, hypotension, muscle cramps, paralysis, and even rarely, death^[8]. In 2013 Uthayasiva *et al.* studied the microscopy observations of collected sting ray fishes and results were exposed that the spine contains epithelial cells with distinct pigmentation, the spines fixed in the fibrous tissue of the dorsal part of the root of tail. The spine built from vasaodentine and covered with a layer of very hard vitrodentine. Similar types of results have been reported by Ravi *et al.*^[16] who have observed the microscopic studies on stingray fish *H. imbricata* collected from Parangipettai coastal region. Liu *et al.* have observed the glandular tissue is also found along the dorsum of the tail below the spine venom of sting ray fishes and the epithelium consist of about 4 layers of cells from the base to the surface. Danielle Tartar *et al.*^[9] explained the histopathological findings in stingray injuries have not been well characterized. A large zone of pauci-cellular necrosis (necrobiosis) with surrounding granulomatous inflammation and superficial ulceration was

present in a biopsy taken 2 months after the injury occurred. This pattern of necrosis may stem from direct toxicity of the stingray venom on the soft tissues of the skin^[14].

Treatment of stingray injuries should have main goal to relieve pain and prevent wound infection and tissue necrosis by deriding the wound and administering if necessary appropriate antibiotics^[3]. This reason for current study was carried to identify the functional group for further pharmacological work. The variation spectrum of a molecule was considered to be a unique physical property and was characteristic of a fingerprint for identification by the comparison of the spectrum from the unknown with previously recorded reference spectra. Over the years, much has been published in terms of the fundamental absorption frequencies which were the key to unlock the structure–spectral relationships of the associated molecular vibration^[19]. In the present study was developed for isolation and identification of the functional compounds present in the stingray spines of three species. Each compound has a characteristic set of absorption bands in its infrared spectrum. Characteristic bands found in the infrared spectrum of proteins and polypeptides include amide I and amide II. These arise from the amino bonds like that of the amino acids. The absorption associated with amide I band leads to stretching vibrations of the C=O bond of the amide, absorption associated the amide–II band leads to primarily bending vibrations of the N–H bond.

Because of both C=O and N–H bonds were involved in the hydrogen bonding that takes place between the different elements of secondary structure, the location of both amide I and amide II bands were structure content of a protein. Studies with protein of known structure have been used to correlate systematically the shape of the amide I bend to secondary structure content^[22]. The result of FTIR spectroscopy revealed the presence of free amino acids and protein having β -sheet and random coiled secondary structure. From the results, it could identify the presence of O–H stretch, C=O stretch, C–H stretch, N–H deformation, O–H deformation and C–O stretch in the sample aligned with standard BSA. Apart from this, there are several functional groups fall outside the quoted ranges. This was to be expected for several reasons. The influence of other functional groups within the molecule was because of the impact of preferred spatial orientation and environmental effects (chemicals and physical interaction) on the molecule^[19]. Compared to standard, sample II consists of two C=O stretches were involved in the hydrogen bonding that takes place between the different elements of secondary structure.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Numbers of the marine animals are able to cause major injury/death to the human. There are no appropriate treating measures for injuries especially sting ray. Therefore, there is a need to focus the study on the toxic spines of sting ray.

Research frontiers

The present research work depicts to identify the functional groups of sting rays spine towards the pharmacological purposes.

Related reports

Haddad *et al.*, 2004 also focused the research on the sting ray toxins for pharmacological purposes. Further, Brisset *et al.*, 2006 have studied the fresh water ray fish towards the pain reliever, prevent wound infection and tissue necrosis.

Innovations and breakthroughs

The result of FTIR spectroscopy revealed that we could identify the presence of the functional and structural groups in the sting ray toxin. The study has focused the innovative and modified methodology to know the functional groups of sting ray toxin for development of toxoid towards the anti-venom preparation.

Applications

From the literature survey, studies on the functional groups of the protein by FTIR technique is one of the easiest and best technique.

Peer review

The venom of poisonous animals has been broadly studied because of their potential source as pharmacological activities. This is the valuable work on the sting ray spines towards the anti-venom synthesis. The study will be helpful for further pharmacological research to develop a treatment drugs or toxoids for poisonous animal injury and other purposes.

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