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Antimicrobial and anticoagulant activities of the spine of stingray *Himantura imbricata*

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PEER REVIEW

Peer reviewer

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Comments

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ABSTRACT

Objective: To study the spine structure of stingray *Himantura imbricata* (*H. imbricata*) and to evaluate the anticoagulant properties of the spine extract obtained through various solvents extracts followed by antibacterial activity against human pathogens.
Methods: Spines of *H. imbricata* were collected from Nagappattinam coast, Tamil Nadu, India and their spines were observed under the light microscope. The grounded spines were subjected to extraction of metabolites using methanol, ethanol, chloroform and acetone. Antibacterial activity was evaluated by disc diffusion technique against 10 human pathogens. Similarly, anticoagulant activity was also assessed by following United States Pharmacopeia method.
Results: Light microscopic observation of spine revealed that the venom apparatus of the stingray *H. imbricata* consisted of two to three spines, glandular tissue and a sheath. The spine extract showed potent antibacterial activity against all tested pathogen. Maximum activity (14 mm) was found against *Staphylococcus aureus*. Crude extract showed 91.50 USP units/mg of anticoagulant activity.
Conclusions: Microscopic observations gave new insight about the spine structure of the stingray. The spine extracts of *H. imbricate* showed potent activity against human pathogens revealed by the good zone of inhibition. Chloroform extracts conferred the most prominent antibacterial activity. The anticoagulant activity was also comparable with that of standard heparin.

KEYWORDS

Stingrays, *Himantura imbricata*, Spine, Antimicrobial activity, Anticoagulant activity

1. Introduction

Marine natural products are endowed with promising antibacterial activities, thus representing invaluable leads in antibiotic drug discovery and are playing pivotal role in medicine and, in particular, marine metabolites, have increasingly becoming major players in recent drug discovery[1]. For the past two decades, pharmaceutical industry has been relatively successful in overcoming problems due to single resistant determinants[2]. However, the advent of multiple resistant mechanism has limited the use of many major classes of antimicrobial compounds. The demand for effective and non-toxic antibacterial therapeutics has become even greater with the increased incidence of bacterial infections. Substances with anticoagulant activity are among the first choices as functional components which are being used to open up new areas of application for anticoagulant pathogens[3].

Ray fishes (Class Chondrichthyes) are bottom-dwelling, free swimming fishes, evolved with a flattened body.

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Stingrays have one or more serrated spines on their whip shaped tail. These serrated spines are covered by an epithelial layer that has venom secretory cells and they are located in the epithelium or in close contact with it[4,5]. Serrated spines of rays may cause mechanical damage in victim's tissues and liberate venom to the injured tissues as well[6]. Stingrays do not attack people, however if they are stepped on, stingrays utilize its spine as a form of defense. When the wings of the ray are touched, its tail whips in response, thrusting the spine into the victim and causing a puncture of wound or jagged laceration. The integumentary sheath overlying the spine ruptures, and the venom is released into the wound along with mucus, pieces of the sting sheath, and fragments of spines. The entire tip of the spine may remain embedded in the wound[7].

There appear to be several chemicals in the venom, but not all of these have been well characterized to date. Anticoagulants play a crucial role as agents for the prevention and treatment of thromboembolic disorder[8]. For more than five decades, anticoagulant drugs consist of heparins, vitamin K-antagonists and their derivatives have been the major players in the clinical setting. Although their efficacy remains undisputed, the deleterious life-threatening side effects of these drugs have also been well documented[9].

Studies on stingrays from Indian waters are in food and feeding habits and length-weight relationships distribution spines ultra structure observation and reproduction[10-13]. The scaly stingray *Himantura imbricata* (*H. imbricata*) is commonly distributed in Nagappattinam coastal waters[11]. Reports on the spine studies of the stingray are scanty. Therefore, the present study was aimed to study the venom properties of stingray *H. imbricata* spines, their antibacterial activity against human pathogens and anticoagulant activities.

2. Materials and methods

2.1. Collection of spine and microscopic observation

Spines of *H. imbricata* were collected carefully, immediately after landing from Nagappattinam coast (10°46′ N, 79°51′ E), Tamil Nadu, India and the spines were rinsed with sterile water to remove associated debris and transferred to sterile polythene zip pouch. Measurements on spines were made to the nearest length (cm) and weight (g). Spines were observed in the light microscope (10×) to measure the length of the barbs with the help of oculometer.

2.2. Solvent extraction of venom

The spine was pulverized in the presence of phosphate buffer saline (pH 7.4). The grounded material was subjected to extraction using methanol, ethanol, chloroform and acetone in the ratio of 2:1[14]. The extracts were then obtained from the soaked samples by grinding, using pestle and mortar and filtering through Whatman No. 1 filter paper. The filtrates were centrifuged at 3 000 r/min for 10 min. The supernatant was collected and used for further studies.

2.3. Antibacterial activity

Antibacterial activity of crude spines extracts of *H. imbricata* was determined against 10 bacterial strains such as *Escherichia coli* (*E. coli*), *Klebsiella pneumonia*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonous aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Staphylococcus aureus* (*S. aureus*), *Vibrio cholera* and *Vibrio parahaemolyticus*. All the pathogenic bacterial strains were obtained from Raja Muthiah Medical College, Annamalai University.

Antibacterial activities were carried out by disc diffusion technique[15]. Areas of inhibited bacterial growth were observed as clear halos zones around the disc. Antimicrobial activity was measured as diameter of zone of inhibition, excluding the paper disc diameter. All experiments were performed in triplicate.

2.4. Anticoagulant activity

The anticoagulant activity of the sample was evaluated using (the whole) sheep plasma by following the United States Pharmacopeia (USP) method. The minimum quantity of heparin sodium (0 to 100 μg) standard was determined by adding 0.8 mL of saline solution which maintains fluidity of prepared plasma for 1 h after the addition of 0.2 mL calcium chloride solution (1 g in 100 mL). This quantity is usually between 1 to 3 USP heparin units.

2.5. Assay preparation

About 25 mg of heparin sodium was accurately weighed and dissolved in 25 mL of saline solution to obtain a concentration of 1 mg/mL.

3. Results

3.1. Spine structure

Light microscopic observation revealed that the venom apparatus of the stingray *H. imbricata* consisted of two spines, glandular tissue and a sheath (Figure 1). The spines are fixed in the fibrous tissue of the dorsal part of the root

of the tail. The spine is built from vasaodentine and is covered with a layer of very hard vitrodentine. Laterally, on the ventral side, there are grooves that contain the glandular tissue, enveloped by the sheath. In the course of the stinging act, the sheath breaks and the venom is mechanically expressed in the wound. Glandular tissue is also found along the dorsum of the tail below the spine. The serrated margins of the spine with barb contribute to breaking the sheath and broadening the wound (Figure 2). The sheath is highly pigmented with dark brown materials.

Figure 1. Spine structure of *H. imbricata*.

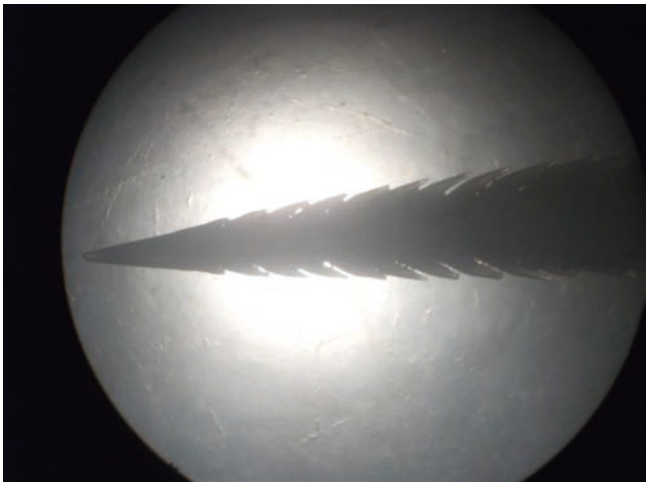

Figure 2. Light microscope structure (10x) of the serrated spine of the *H. imbricata*.

3.2. Antibacterial activity

Four solvent extracts (acetone, chloroform, ethanol and methanol) of sting ray crude spine were tested against 10 human pathogens by disc diffusion methods (Figures 3 and 4) in the present study. The results of the screening test are summarized in Figure 5. Among the tested samples, maximum activity (14 mm) was recorded against *S. aureus* and minimum activity (1 mm) was in *E. coli*. Most prominent antibacterial activity was conferred by the chloroform extracts.

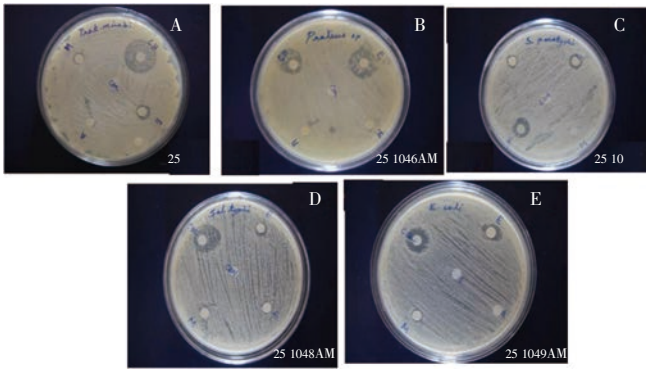

Figure 3. Diameter of inhibition zone of stingray *H. imbricata* crude spines against each test microorganism (A–E).

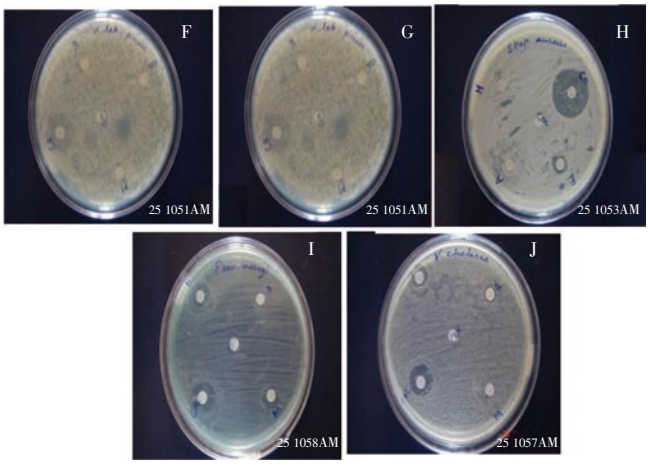

Figure 4. Diameter of inhibition zone of stingray *H. imbricata* crude spines against each test microorganism (F–J).

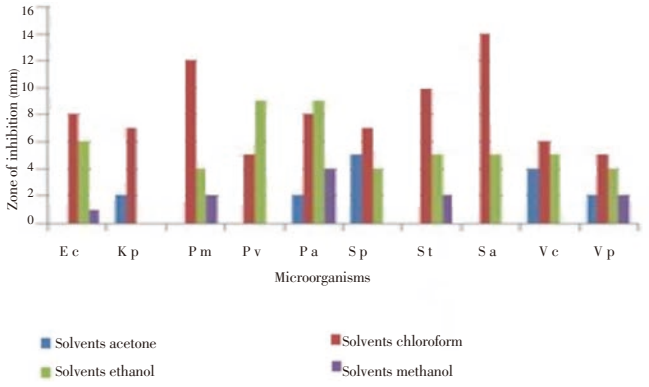

Figure 5. Effect of *H. imbricata* crude spines against each test microorganism. E c: *Escherichia coli*; K p: *Klebsiella pneumonia*; P m: *Proteus mirabilis*; P v: *Proteus vulgaris*; P a: *Pseudomonous aeruginosa*; S p: *Salmonella paratyphi*; S t: *Salmonella typhi*; S a: *Staphylococcus aureus*; V c: *Vibrio cholera*; V p: *Vibrio parahaemolyticus*.

3.3. Anticoagulant activity

Anticoagulant activity was tested according to the UPS methods. Crude extracts from *H. imbricata* showed the anticoagulant activity of 91.50 USP units/mg, whereas in the case of standard heparin saline the activity was found to be 108.10 USP units/mg.

4. Discussion

Venom of poisonous animals has been extensively studied for their potential source of pharmacological agents and physiological tools. During the course of evolution, venomous animals developed highly specialized and sophisticated strategies basically serve for prey capture and/or defense purpose. Recently, Rethna Priya *et al*. has studied the bioactive proteins from pipefishes[16]. Despite there has been much work characterizing the biological activity of the most terrestrial animals (e.g. snakes, spiders, and scorpions), comparatively less attention have been paid on venomous ray fish. Fish toxins represent a vast source of novel pharmacological compounds that may prove to be useful research tools and therapeutic agents.

The main difficulty in the study of stingray venom is that these fish lack true venom glands so that the venom is obtained only by the maceration of glandular tissue[17]. The microscopy observations revealed that spine contained epithelial cells with distinct pigmentation. The spines were fixed in the fibrous tissue of the dorsal part of the root of the tail. The spine was built from vasodentine and covered with a layer of very hard vitrodentine. Laterally, on the ventral side, there are grooves that contain the glandular tissue, enveloped by the sheath[12]. In the course of the stinging act, the sheath breaks and the venom is mechanically expressed in the wound. Clinical and histopathologic findings have been also reported in cutaneous sting ray wounds[18].

The antibacterial activity was tested against ten human pathogens by paper disc diffusion methods. Among the tested samples, maximum activity (14 mm) was found in *S. aureus* and minimum activity (1 mm) was in *E. coli*. The most prominent antibacterial activity was conferred by the chloroform extracts[19]. Antimicrobial and proteolytic property of the epidermal mucus secretion of marine stingrays revealed that the acidic mucus extracts have potential antimicrobial activity, indicating that antimicrobial peptides or acidic soluble proteins are responsible for the defensive purposes against the invading pathogens. Screening of antibacterial drugs from marine gastropod *Chicoreus ramosus* has also been done most recently by Ramasamy *et al*[20]. Stingray venom is a potential source of novel bioactive peptide like Orpotrin and Porflan[21]. Prabhu *et al*. (2013) demonstrated the crude and fractions of starfish *Stellaster equestris* have remarkable antimicrobial activities against human bacterial pathogens[22].

Crude extract showed 91.50 USP units/mg of anticoagulant activity compared to 108.10 USP units/mg with that of standard heparin saline. The anticoagulant activity might be due to the presence of fibrin (ogen) olytic enzyme[23]. The studies on extracts of tissue covering stingers of marine and freshwater stingrays (*Dasyatis gutata* and *Potamotrygon falkneri*) showed anticoagulant activities, edematogenic, gelatinolytic, caseinolytic and fibrinogenolytic activities reported that anticoagulant activity of crude sample from molluscan species ranged from 70 to 120 USP units/mg whereas the activity ranged from 130 to 150 USP units/mg after purification[24-26].

To conclude, microscopic observations gave new insight about spine structure. The spine extract showed potent activity against human pathogens revealed by the good zone of inhibition. Chloroform extracts conferred the most prominent antibacterial activity. The anticoagulant activity was also comparable with that of standard heparin. Hence, based on the above finding, it is envisaged that the stingray spine could be an alternative to the mammalian heparin and stingray can be a potential source of antimicrobial compounds. However, further studies on epidemiology, venom properties, clinical findings especially systemic manifestations and the necessary treatment are required for a better approach.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

For the effective and non-toxic antibacterial therapeutics, marine natural products are recently being explored extensively. In this context, the present research is of utmost importance emphasizing on both anticoagulant and antibacterial properties of sting ray spines.

Research frontiers

The author has studied the venom properties of sting ray *H. imbricata* spines, their antibacterial activity against human pathogens followed by anticoagulant activities. The anticoagulant activity was also comparable with that of standard heparin. The spine extract showed potent activity against human pathogens revealed by the good zone of inhibition. Chloroform extracts conferred the most prominent antibacterial activity.

Related reports

Stingray venom is a potential source of novel bioactive peptide like Orpotrin and Porflan. The studies on extracts of tissue covering stingers of marine and freshwater

stingrays (*Dasyatis gutata* and *Potamotrygon falkneri*) have been reported to show anticoagulant activities, gelatinolytic, caseinolytic and fibrinogenolytic activities.

Innovations and breakthroughs

Substances with anticoagulant activity are among the first choices as functional components which are being used to open up new areas of application for anticoagulants pathogens. Although there are several attempts from various marine organism to assess the anticoagulant and antibacterial properties. But, this paper assessed the properties from sting ray spine.

Applications

Of course the present work paves the way for further research. The anticoagulant properties can be employed for various medicinal purposes. However, further purification and screenings will be required.

Peer review

Reports on the stingray spine for its anticoagulant and antibacterial properties are scanty. Crude extract showed 91.50 USP units/mg of anticoagulant activity. The spine extract showed potent antibacterial activity against all tested human pathogen.

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