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Ravi Gugulothu
Teaching Faculty, College of Fisheries Science, Pebbair, Wanaparthy, Telangana, India

Balaji Guguloth
Fisheries Scientist, Krishi Vigyan Kendra, Mamnoor, Warangal Urban, P V Narsimha Rao Telangana Veterinary University, Telangana, India

Srinu Rathlavath
Teaching Faculty, College of Fisheries Science, Pebbair, Wanaparthy, Telangana, India

Koteswar Banoth
PQAS NIFPHATT, Government of India, Vishakhapatnam, Andhra Pradesh, India

Raveendar Banothu
Teaching Faculty, College of Fisheries Science, Pebbair, Wanaparthy, Telangana, India

Correspondence
Ravi Gugulothu
Teaching Faculty, College of Fisheries Science, Pebbair, Wanaparthy, Telangana, India

Bioactive properties of *Conus betulinus* collected from Thoothukudi coast of Tamil Nadu India

Ravi Gugulothu, Balaji Guguloth, Srinu Rathlavath, Koteswar Banoth and Raveendar Banothu

Abstract
The genus *Conus* represents the toxic gastropod animals, with venomous gland producing a cocktail of peptides referred as conotoxin. These conotoxins has immense bioactive properties. In this study, *Conus betulinus* (Gastropod) collected from Thoothukudi of Tamil Nadu. Protein content in the crude toxin was 1320±15 µg/ml by Lowry method. Analysis of crude venom extract on SDS page analysis indicated three prominent bands at 35 kDa, 41 kDa and 90 kDa region. Increase in Acetyl Choline Esterase activity was observed due to addition of *Conus betulinus* crude venom extract at all the concentrations. Analgesic activity studied through Eddy's hot plate method indicated highest analgesic effect due to i.p. injection of venom toxin at 0.25 ml. Characterisation of crude extracts indicated that marked property reduction due to addition of acid and alkali. The effect of *Conus betulinus* venom on isolated heart preparation was studied at three concentrations of 10µg, 20µg and 40µg. *Conus betulinus* venom extract at 20µg shows increase in the amplitude of contraction. Through this study we concluded that *Conus betulinus* has neuromodulatory property.

Keywords: conotoxin, bioactive properties, *Conus betulinus* venom, Radular teeth

1. Introduction
Conidae is one of the major group of gastropod animals represent the super family Toxoglossa, which are mainly characterized by the possession of intense venom apparatus. These cone shells can sense the presence of prey through the passage of water odours to its chemoreceptory organ [8]. These predacious gastropod cone shells have a diverse mechanism of prey capture, is into three major feeding types namely; piscivorous, vermivorous, and molluscivorous [9]. Among these feeding types, piscivorous species are very dangerous, these species have the ability to kill and swallow the prey of similar size [8]. Irrespective of the feeding type of *Conus* species, and the conotoxins, these species have a similar venom apparatus [1].

The venom apparatus of cone snail consisting of venom bulb, venom duct and radular sheath. They lie in a cavity within the animal and believed that preparatory to stinging, the radular tooth that are housed in the radular sheath and then released to the pharynx after that enter into the proboscis, where they are grasped for thrusting into the flesh of the victim. The venom is produced in the venom duct by the contraction of venom bulb and thereby forced into the coiled radular teeth. The venomous gland produces neurotoxic peptides and is a cocktail of different peptides referred as conotoxins [14]. It acts mainly on ion channels such as Ca, Na and K ion channel. These will affect the Central Nervous System, and thereby ultimately lead to death. The venom of each species of *Conus* has estimated to comprise between 100-200 peptide components [12]. In the last two decades, substantial progress has been made in elucidating components of cone snail venoms [13, 14]. The characterization of cone snail venoms has a significant impact on toxinology, since the studies that have revealed the mode of action of peptides from these venoms has led not only to understanding basic mechanisms that underlie *Conus* envenomation, but also to significant application in neurobiology and other biomedical sciences. A few cone snail venom components are even under development as the therapeutic agents [16]. A unique feature of conotoxins is due to their high degree of post-translational modification which is up to 75% of the amino acids in a single conotoxin found to be modified [5]. McIntosh *et al*., (2001) [11]. Reported the presence of serotonin, a smooth muscle relaxation compound in the venom of the Cone snail, *C. imperialis*. Studies on conotoxin are very much limited in India. Especially, in the south east coast of India.

Considering the abundance of *Conus betulinus* in Thoothukudi coast and its venom is one of the treasure houses of the pharmacological active compounds. This study was decided to investigate the spasmogenic action of *C. betulinus* venom on vertebrate, smooth, skeletal and cardiac muscles with the view to elucidate the pharmacological mechanism involved.

2. Materials and Methods

2.1 Preparation of venom from *Conus betulinus*

Live *Conus betulinus* were collected from Tuticorin coast. Shells were cracked and the animals were removed from the shells. The venom duct of each animal was dissected out. The anterior end of duct was cut open at its junction with pharynx and the posterior end of the duct was at its junction with venom bulb. Venom was stripped from the venom duct by the method provided by Endean *et al*., (1974) [3]. Stripped venom was then weighed and taken up and mixed with 1 ml of 0.85% saline solution. Saline extracts were then freeze dried and stored in a sealed ampoules. The freeze dried material was weighed and taken up with physiological saline before use.

2.2 Test 1: Behavioural studies of mice on *Conus betulinus* venom administration

Behaviour of swiss wistar albino mice on oral and intraperitoneal administration was observed at different concentrations. Their activities were recorded along with controls.

2.3 Test 2: Acute lethality test

The LD_{50} value was calculated by using saline extracts of freeze dried venom by method of Horn, (1956). Injected animals were carefully observed for 48 hours prior to death in both oral and intra peritoneal route at different concentration.

2.4 Test 3: Characterisation of *Conus betulinus* venom

The effect of heat, light, storage, pH and freeze- drying of *C. betulinus* venom were studied. The action of venom with various pH and enzyme were studied to analyse the chemical natures and stability of venom. After the incubation period the stability of venom was tested by pharmacological experiments.

2.5 Smooth muscle preparation

Rabbits of either sex were killed by cervical dislocation. The ileum was taken out and placed oxygenated tyrode solution (137 mM NaCl, 27mM KCl, 1 mM $MgCl_2$, 0.4 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 1.2 mM $NaHCO_3$, and 5.5 mM $C_6H_{12}O_6$:). In order to minimise slow movement, short segments were used. This preparation equilibrated at passive tension, until study state was achieved. Stability were also tested by the induced contraction by acetylcholine, selected viable preparation were used to execute the tests.

2.6 Isolated heart preparation

Hearts were dissected out from healthy frogs and mounted vertically in an organ bath containing oxygenated ringer solution (115 mM Nacl, 2.5 mM Kcl, 1.8 mM $Cacl_2$, 2.15 mM Na_2Hpo_4, 0.85 mM NaH_2PO_4, and 5.5 mM $C_6H_{12}O_6$). Stability and viability of this preparation were tested by adding minimum dose of adrenaline contraction elicited from selective and viable preparation were recorded in Kymograph

3. Results

Behavioural studies of albino mice on oral and intraperitoneal administration of *C. betulinus* venom at two different concentrations 5µg/kg and 7µg/kg observed very carefully for 48 hrs with control saline treated mice. The action of venom greatly demolished on oral administration. Animals administered orally were normal up to 18 minutes. At the beginning of 20th minute, it was slightly restless and respiratory rate was sharply increased. At the onset of 30th minute animals tends to take rest with palpitation. At the onset of 40th minute animal become perfectly normal. No remarkable difference were observed in the behaviour of control and treated. But as a whole no serious problem was encountered by mice on both dosage of oral administration until 48 hrs.

Intraperitoneal administration of *C. betulinus* venom at two different concentration i.e., 40µg and 50µg per gram body weight shows normal activity up to 5th minute. There were no remarkable changes in their usual behaviour when compared to control. At the beginning of 7th minute respiratory rate was gradually increased with some occasional tremor. At the end of 10th minute they were become totally restless, respiratory rate was sharply increased with some an unusual behaviour like dragging of limb with very occasional but very strong tremor. At the end of 14th minute dragged their oedematous limb and refused to move the limb while provoked. At the onset of 27th minute respiratory rate was markedly increased, they tried to drag the limb but failed. At the end of 43 minute respiratory rate was sharply increased, severe tremor was observed. They could not hold their head properly due to the initiation of skeletal muscle paralysis. While provoking, there was sharp delay in a speed of reflux. They failed to respond for even pinpricks. Both entered into coma state within an hour. At the end of one hour thirteen minute after intraperitoneal administration, death occurred due to cardiac failure. The above results were confirmed with triplicate. Saline extracts of *C. betulinus* venom maintained at 60 °C for an hour shown no loss of activity whereas venom maintained at 100 °C for an hour shown 50% loss of activity and the venom maintained at 100 °C temperature for 3 hours shown complete loss of activity. Action of *C. betulinus* venom on smooth muscles at 40 – 50 µg level gives sharp contraction and shows agonistic activity to acetylcholine at 4µg level. This action was not blocked by any respective blockers of muscrinic receptors or cholinergic receptors. Action could be recovered by repeated washing. The contraction action of venom is dose dependent. An effect of venom has not blocked by atropine sulphate at 40µg level in both frog rectus muscle and rabbit ileum.

Figure 1 illustrates the effect of *Conus betulinus* venom on isolated heart preparation at the concentration of 10µg, 20µg, 30µg and 40 µg. *Conus betulinus* venom at 20µg shows remarkably increase in the amplitude of contraction. At the maximum concentration 40µg shows complete cardiac depressant action. Figure 2 clearly illustrates the antagonistic action of venom is purely dose dependent. Neither propranolol nor tolozulol have block the action of *Conus betulinus* venom clearly reveals that the effect of venom not through the appropriate blockers. The action is through neuromuscular receptor blocking action like d-tubocurarine (DTC).

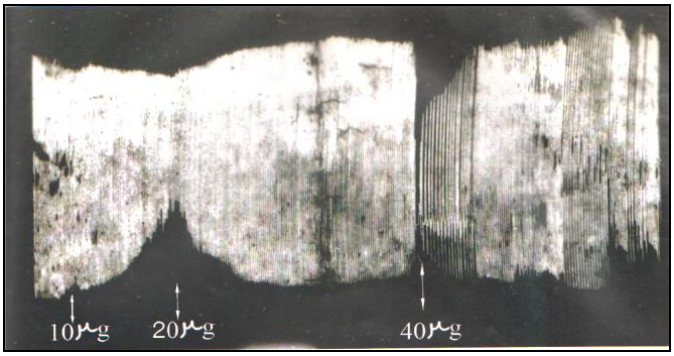

Fig 1: *Conus* venom (*C. betulinus*) induced cardiac depressant activity on isolated frog heart

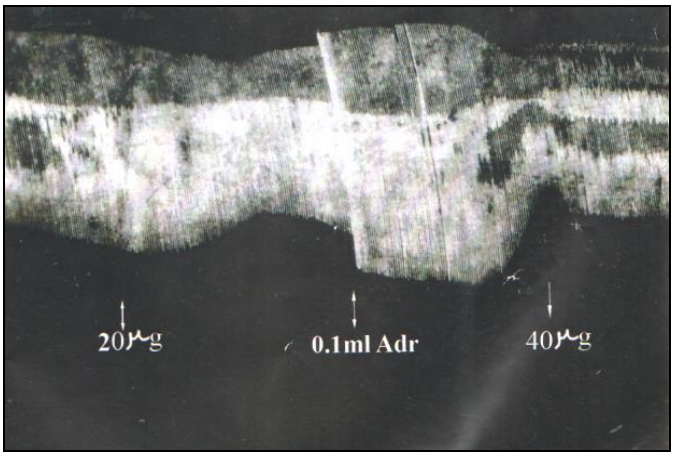

Fig 2: Adrenalin induced increased amplitude in isolated frog heart

4. Discussion

Absence of labelin gland and its evolutionary modifications as venom apparatus could be considered as a distinctive diversification of this genus. Most of the work focused on the venom apparatus and their active secretion mainly based on the observation of man killing piscivorous cones. There is no morphological diversification of venom apparatus exists in piscivorous, molluscivorous and vermivorous cones. Even though all three types of cones available in Indian waters, vermivorous cones are predominant over than other two.

LD_{50} value of venom showed the amount of venom required to inactivate the polycheate worms was comparatively lesser than gastropods and fishes. Venom from few *Conus* species is not much toxic to human being but it can cause some local damage. The real threat to human beings is from piscivorous type cones, these display attractive colour pattern to lure the prey. In the present study at minimum concentration of 40 µg/kg *C. betulinus* venom subdue their active prey. Venom of two piscivorous cones showed two different pharmacological properties reveals the presence of more than one active compound. So many active peptides like omega conopeptides, alpha conopeptides and shaker peptides have been isolated from *Conus* venom by Olivera *et al*., (2007) [15]. In the present experiment results clearly illustrate *C. betulinus* venom exhibits spasmogenic action through adrenogenic receptor. But the action of the venom may either directly or by gangliogenic blocking mechanism. Agonistic action of Conus venom with known spasmogen on smooth muscles was well reviewed by Endean *et al*., (1977) [4]. Action of agonists suggested that *C. betulinus* venom contained analogous transmitters like acetylcholine, serotonin and histamine. But unfortunately the effect of *C. betulinus* venom was not abolished by corresponding blockers. This inhibitory action of *C. betulinus* venom on smooth muscles also supported by Maggi *et al*., (1994) [10]. However, studies on pharmacological action of crude venom will be complicated by the presence of more than one active compound.

Inertness of the *C. betulinus* venom during oral administration clearly indicates that the active compound present in the venom are basically proteins. They may get inactivated by proteolytic digestive enzymes. It also shows close similarities with curari form drugs on i.p. administration. *C. betulinus* venom on smooth muscles suggested that the action of venom shows close analogy with d-tubocurarine (DTC). The principle action of venom on neuromuscular junction and cause muscular paralysis clearly reveals the presence of paralytic peptides analogous to curare form drug. The partial agonistic action of this venom with acetylcholine induced contraction suggested that it might be due to the presence of analogous transmitters which cause peristaltic movement during fast venom ejaculation. The effect of *Conus* venom on cardiac muscles suggested that the action of venom may be due to the presence of cardiotonic glycoprotein as shown by Kobayashi e*t al*., (1982) [6], and also evidenced by victims. In serious cases skeletal muscle paralysis followed by respiratory failure and death occur due to cardiac arrest. Figure 1 clearly shown that the action of venom on cardiac muscles was purely dose dependent. The action of venom on neuromuscular junctions appears to be essential if the mollusc preyed on active fishes. So it is not surprising that the venom acts through neuromuscular junction as shown by Endean *et al.*, (1963) [2].

C. betulinus venom is a complex of lethal and non-lethal peptides which assists prime compounds for rapid distribution. It has been well investigated the principle action of venom mostly through cardiac muscles by preventing the action of adrenaline and preventing the acetylcholine in the mode of curare form drugs. Being multiple neurotoxic peptides it shows close similarity to the snake venom. Subcutaneous administration of adrenaline can be very useful to stabilise the heartbeat. In severe cases artificial respiration is very useful with adrenaline therapy.

5. Conclusion

Though terrestrial organisms has given more active drugs like captopril- an antihypertensive drug from snake venom. Being a major phyla mollusc holds prime important in the field of biomedical research. Present mark is an out growing our interest in malacology and marine pharmacology. This study on *C. betulinus* may provide novel pharmacological and physiological insight into mechanism of neurotransmission. An attempt has been made to diagnose the mode of action of venom by using adrenaline and acetylcholine with its respective blockers. Through this study we concluded that the cone, *C. betulinus* has neuromodulatory property.

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