Toxicity and histopathological observations on albino mice on intra-peritoneal injection of three species of Conus
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INTRODUCTION
The bioactive components of Conus venom are mostly small peptides that typically are 10-30 amino acid residues in length and each of the species of cone snails has its own distinctive complement of venom peptides (Olivera et al., 1991). The toxin composition of Conus venom is
species specific. Similar or nearly identical constituents possibly exhibit distinct biological activities, considered worthy of clinical application (Jones & Bulaj, 2000; Livett et al., 2004). Compounds of medical interest are likely to be present in all venoms, regardless of the size of the animal. In particular, cone snail venoms contain mostly small and highly structured peptides called conopeptides (Olivera et al., 1990) that show an extensive diversity of pharmacological activities (Favreau & Stocklin, 2009). These peptides have attracted much interest due to their high affinity and selectivity toward a large array of therapeutically relevant targets (Lewis & Garcia, 2003). Already one marketed analgesic drug, Ziconotide or Prialt, has emerged from conus research (Miljanich, 2004), it is estimated that only 0.1% of the total venom diversity has been uncovered (Lewis, 2009). However, interest has been extended beyond this into the development of novel bioactive compounds of therapeutic importance. Therefore, it seems Conus venom peptides exhibit a diverse pharmacology of peptides that potently affect the function of voltage-gated ion channels (Uchitel, 1997). Conus peptides have also been directly developed as drug candidates that have most promising anti-convulsant properties compares to the commercial antiepileptic medications currently available in the market (Olivera et al., 1995). There is tremendous level of worldwide interest in marine natural products with therapeutic potential in industry, academia and government research laboratories, largely because natural products generally continue to be viewed as one of the sources of drug discovery, yielding unorthodox and often unexpected chemical structures that offer novel points of departure for molecular modification leading to clinically available drugs (McConell et al., 1994). Several postulates have been presented to explain the generation of conotoxin diversity (Olivera et al., 1990; Craig et al., 1999; Espiritu et al., 2001; Dutton et al., 2002).

Histopathological alterations have been used as markers to better understand animal health exposed to contaminants in lab (Wester & Canton, 1991; Thophon et al., 2003) and field studies (Hinton et al., 1992; Schwaiger et al., 1997; Teh et al., 1997). The main advantages of using histopathological markers in monitoring is that this category of markers allows to study the target organs, including brain, heart, kidney and liver, that are responsible for important functions, such as excretion and the deposition and bio-magnifications of toxins in the fish (Gernhofer et al., 2001). The changes found in these organs are normally as general to pinpoint than functional ones (Fanta et al., 2003), and exhibit as alarming signals of alterations to general animal health (Hinton and Laurén, 1990).

Therefore, the present study was undertaken on three cone snails Conus inscriptus, Conus lentiginosus and Conus zeylanicus with reference to their pharmacological potential may be useful for human health in year to come. However, the effect of these venoms in mice have not been evaluated, through evaluation of brain, heart, kidney and liver tissues and, therefore, was the main interest of present study.

MATERIALS AND METHODS

Collection of sample
The samples of Conus sps. were collected from Khardanda shore, Mumbai. The specimens were kept alive in salt water aquaria until needed. A total amount of 2.5 g of crude venom was extracted from about 40-50 each
specimens. Venom was extracted from freshly sacrifice animals as described by Cruz et al (1976). The soft body of the animal was removed by breaking the shell. The venom duct and venom bulb of each animal was dissected out. The venom duct was grinded and mixed with double distilled water (DDW). The grind ducts were centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected for lyophilisation and stored immediately at 4°C. The lyophilized powder was re-suspended in DDW and it was considered as crude venom. The extracts were stored at -20°C for further analysis.

**Experimental Animal**

Male albino mice of Kausauli strain weighing 20 ± 2 g were procured from the animal house of M/S Haffkine Bio-pharma, Mumbai, and were maintained in the laboratory, following the codal formalities of the Institute’s Ethical Committee.

**Partial Purification of Toxin**

Partial purification of the crude extract was carried out through DEAE cellulose anion exchange chromatography, following the method of Shiomi et al (1987) and protein was estimated by Lowry et al. (1951)

**Mice Bioassay for Lethality**

The mice bioassay was carried out according to method Gouiffes et al. (1988). The crude venom dissolved @ 5 mg/mL in PBS was injected intraperitoneally (i.p.) to the mice in doses of 1.0 mL of each fraction was injected i.p. to the mice. Triplicate sets were maintained for each dose. The injected mice were kept under observation in mice rearing cages. The time of injection and the time of death were recorded, besides recording the behavioral changes before death.

**Autopsy**

Mice that died upon envenomation were autopsied to observe gross anatomical changes, if any, such as hemorrhage, blood clots, septicemia, dark or pale discoloration of internal organs, etc. Autopsy was conducted on test mice, which died upon envenomation to observe gross anatomical changes such as hemorrhage, granular appearance and or discoloration of brain, heart, liver, kidney, septicemia and other abnormalities.

**Histopathological Study**

Brain, heart, liver and kidney were dissected out from mice that died upon en-venomation while ascertaining the toxicity of the Coniid extracts. The dissected organs were fixed in 10% formalin for a minimum period of 24 hours and further processed according to Lefkowitch (1987). Prepared section were examined and photographed under a light microscope.

**RESULT**

**Protein Estimation**

Protein content in the crude toxin was 342.0 μg/mL in C. inscriptus, 432.5 μg/mL in C. lentiginosus and 281.6 μg/mL in C. zeylanicus. Protein content in the purified toxin was obtained from C. inscriptus ranging from 60.5 - 219.8 μg/mL, C. lentiginosus 80.2 - 281.2 μg/mL and C. zeylanicus 52.8- 192.4 μg/mL respectively (Table 1, 2 and 3).

**Mouse Bioassay for Lethality**

Protein content of the three Conus species when injected i.p. into male albino mice (20 ± 2 g) showed mortality. In the present study of C. inscriptus showed four purified of lethal fractions were identified, when 1.0 mL of purified toxin was injected. Lithality was observed in mice injected i.p. with venom in C. inscriptus F2 containing 208.4 μg/mL of protein showed lethality in 1 min and 15 sec, F4 containing 179.4 μg/mL of protein in 1 min and 42 sec, F7 containing 151.1 μg/mL of protein in 2 min and 30 sec and fraction 9 containing 92.6 μg/mL of protein in 2 min and 24 sec (Table 1).
TABLE: 1. The toxicity of purified protein fractions from *C. inscriptus* on male albino mice.

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Protein content (μg/mL)</th>
<th>Death time (Hr:Min:Sec)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>342.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>219.8</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F2</td>
<td>208.4</td>
<td>00:01:15</td>
<td>Lethal</td>
</tr>
<tr>
<td>F3</td>
<td>198.6</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F4</td>
<td>179.4</td>
<td>00:01:42</td>
<td>Lethal</td>
</tr>
<tr>
<td>F5</td>
<td>175.2</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F6</td>
<td>171.1</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F7</td>
<td>151.1</td>
<td>00:02:30</td>
<td>Lethal</td>
</tr>
<tr>
<td>F8</td>
<td>142.5</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F9</td>
<td>92.6</td>
<td>00:02:24</td>
<td>Lethal</td>
</tr>
<tr>
<td>F10</td>
<td>60.5</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
</tbody>
</table>

In the case of *C. lentiginosus*, of two lethal purified fractions were identified. F3 containing 222.6 μg/mL of protein showed lethality in 50 min and 20 sec and F8 containing 152.3 μg/mL of protein in 1 hr, 50 min, 42 sec (Table 2).

TABLE: 2. The toxicity of purified protein fractions from *C. lentiginosus* on male albino mice

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Protein content (μg/mL)</th>
<th>Death time (Hr:Min:Sec)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>432.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>281.2</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F2</td>
<td>243.7</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F3</td>
<td>222.6</td>
<td>00:50:20</td>
<td>Lethal</td>
</tr>
<tr>
<td>F4</td>
<td>209.5</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F5</td>
<td>191.3</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F6</td>
<td>183.8</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F7</td>
<td>162.3</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F8</td>
<td>152.3</td>
<td>01:50:42</td>
<td>Lethal</td>
</tr>
<tr>
<td>F9</td>
<td>122.6</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F10</td>
<td>80.2</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
</tbody>
</table>

In the case of *C. zeylanicus* one purified fraction is lethal, F7 containing 121.6 μg/mL of protein showed lethality in 3 hrs, 20 min and 48 sec (Table 3).
TABLE: 3 The toxicity of purified protein fractions from *C. zeylanicus* on male albino mice

<table>
<thead>
<tr>
<th>Fraction no</th>
<th>Protein content (μg/mL)</th>
<th>Death time (Hr:Min:Sec)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>281.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>192.4</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F2</td>
<td>178.6</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F3</td>
<td>166.3</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F4</td>
<td>159.3</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F5</td>
<td>152.3</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F6</td>
<td>135.7</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F7</td>
<td>121.6</td>
<td>03:20:48</td>
<td>Lethal</td>
</tr>
<tr>
<td>F8</td>
<td>99.8</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F9</td>
<td>72.3</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
<tr>
<td>F10</td>
<td>52.8</td>
<td>-</td>
<td>Non-lethal</td>
</tr>
</tbody>
</table>

**Gross Anatomical Changes**

Blood clots in the anterior body cavity were clearly visible with eye and the liver appeared to be swollen in a few cases of mice injected with venom of *C. inscriptus*. Autopsy of test mice that died upon en-venomation revealed the impact of venom on the brain, heart, liver, and kidney which was further supported by histological observations. Dark discoloration and granular appearance of the heart was noticed in mice injected with the venom of all the three species, whereas the liver did not show any discoloration or other changes.

**Histopathological Changes**

**Brain:** Small maleic focus in the cerebellum of the mice injected with *C. lentiginosus* venom could be seen. Glial nodule formation was also observed in some areas of cerebellum. Venom of the other two species did not produce any detectable change in the brain (Figure I).

![Figure I: Histopathological changes in (A) *C. inscriptus* (B) *C. lentiginosus* and (C) *C. zeylanicus* caused by various venoms in the brain of male albino mice.](image)

(A)  (B)  (C)
Kidney: The changes observed in kidney includes, widespread diffused hemorrhagic area could be noticed in the cortico-medullary junction in case of the mice injected with *C. lentiginosus* venom. Linear hemorrhages were evident at the medullary region. The cortical region also showed accumulation of edema fluid. Glomeruli exhibited no detectable change. Occasional shrinkage in *C. inscriptus* and *C. zeylanicus* of the glomerular tuft could be seen besides wider areas of hemorrhage throughout the kidney parenchyma. However, the cortex did not show any specific change (Figure II).

Liver: Distinctive changes noticed in the liver. Histopathological changes in the mice injected with *C. lentiginosus* venom mice revealed many changes as compared to the control mice. Hepatic cells were disrupted. Central veins in most of the cases were occluded by hemolyzed blood. Perivascular area showed cellular reactions forming granulomatous lesions. However, no vacuolation could be seen. In *C. inscriptus* and *C. lentiginosus* hyperplasia of cortical cells was detected with constriction of sinusoidal space. The hepatic cells showed marked pyknosis of the nuclei and extremely vacuolated areas with honey-comb like appearance in case of mice injected with the venom of these three species (Figure III).

Heart: Venom of all the three species studied had a pronounced cardiotoxic effect. Myocardium revealed myofibrillar separation in a few areas. Mild myocardial hemorrhage could be seen in focal areas without any cellular reactions. Myocardial vessels revealed thrombosis. Large areas of hemorrhage could be seen in the myocardium. Mild accumulation of leukocytes could be observed in the myocardium without any degenerative changes.
of myofibrils. Pericardium did not show any change. Myocardial hemorrhage with hemolysed or partially hemolysed blood was also discernible (Figure IV).

Figure IV. Histopathological changes in (A) C. inscriptus (B) C. lentiginosus and (C) C. zeylanicus caused by various venoms in the heart of male albino mice

DISCUSSION

Toxicity

Among the three Coniids, the crude venom of C. zeylanicus was found to have the least minimum lethal dose followed by C. inscriptus and C. lentiginosus (Table 1, 2 and 3). The number of lethal fractions obtained was more in C. inscriptus (four) when compared to C. lentiginosus (two fractions) and C. zeylanicus (one fraction). As in the present investigation, instances of toxicity of various Coniids have been well established. Lewis & Garcia (2003) have stated that a number of Coniids exhibited toxicity on insects and fishes. Bingham et al. (1996) showed that Coniid venom composition varies among different species.

Gel filtration of C. betulinus venom yielded 12 peaks at 280 nm absorbance (Nallathambi,1993). Fractionation of C. striatus venom by gel filtration with Sephadex G-100 yielded 7 peaks of 280 nm absorbance. HPLC analysis of C. monile, C. inscriptus yielded 5 and 4 peaks respectively. Most of the activity seemed to be concentrated in the last 2 major peaks, which produced symptoms like increased palpitation, hyperactivity, violent jumping and convulsions before death indicating potent CNS activity. In the present study, the death time varied between 1 min: 15 sec and 2 min: 30 sec; in C. inscriptus, 50 min and 20 sec and 1 hr: 50 min: 42 sec in C. lentiginosus, while in case of C. zeylanicus death time was 3 hr: 20 min: 48 sec. Nallathambi(1993) reported that only the fractions, III and V were able to elicit any symptoms in mice by intracerebral injection (i.c.) with most of the activity concentrated in I and V which produced shaking, convulsions and sleep like activity. The death time varied between 45 and 55 min and 60 sec in case of fraction I upon i.p. injection. With more than 700 species of Conus, each producing unique sets of hundreds of peptides, there is a tremendous potential for discoveries. However, the isolation of a particular bioactive compound from a complex mixture such as that obtained from a dissected conus venom duct can be a daunting task.

Histopathology

The histopathological studies done by Nallathambi (1993) involving the venom of the cone snail, C. lentiginosus showed that the main organs in mice affected were the heart, kidney and lungs, similar to the findings of Singaravadivelan (2001) using the venom of C. inscriptus. In conformity to these findings, the three venoms affected the heart in the present.
study whereas severe damage was found in the kidney tissue, which confirmed that the target organ was kidney and primarily affected by the toxin, followed by the heart and the liver. Occasional shrinkage of the glomerular tuft could be seen in few places in kidney of mice envenomated with venom of *C. inscriptus* and *C. zeylanicus*. The cortical region in case of mice challenged with venom of *C. zeylanicus* showed accumulation of edema fluid. Alnaqeeb *et al.* (1989) reported that the extensive hemorrhages observed in both the liver and lung tissues could be largely responsible for the tissue destruction caused in these organs due to diminished blood supply leading to necrosis. The section of liver treated with the venom of *Conus inscriptus* showed that the architecture of the liver tissues was disrupted due to severe hemorrhagic necrosis ans was reported by Singaravadivelan (2001) also. A fast acting factor or factors in the venom possibly increased the permeability of the capillaries, causing massive bleeding in the liver and lung (Singaravadivelan, 2001). The histopathological observations in the current investigation are in conformity with these earlier findings. There must have been total hepatic failure since very few normal cells were found in the liver during the present study.

Nallathambi (1993) observed that the venom of *C. betulinus* caused endothelial damage in the heart resulting in the formation of a clot in the ventricular chamber of the heart. Shanmuganandam (1995) reported that coagulative necrosis was observed in the mouse heart treated with *C. figulinus* venom. Singaravadivelan (2001) did not observe any damaging effect on the cardiac tissue treated with *Conus* venom. Myocardial vessels revealed thrombosis besides hemorrhage and severe congestion in the blood vessels. Symptoms like gasping and palpitation shows that the venom in the present study had an impact on the cardiovascular and respiratory systems. Mouse treated with fraction of the *C. lentiginosus* venom revealed glial nodule formation in the cerebrum and small malecic focus in the cerebellum. Whereas, venom from the other two species did not produce any detectable change in the brain. Venom of all the three species studied had a pronounced cardiotoxic effect. Myocardium revealed myofibrillar separation in a few areas. Mild myocardial hemorrhages could be seen in focal areas without any cellular reactions. Myocardial vessels revealed thrombosis. Large areas of hemorrhage could be seen in the myocardium. Mild accumulation of leukocytes could be observed in the myocardium without any degenerative changes of myofibrils. Pericardium did not show any changes. Myocardial hemorrhages with hemolysed or partially hemolysed blood was also discernible. *C. lentiginosus* injected mouse show disruption of hepatic cells. Central veins in most of the cases were occluded by hemolysed blood. Blood vessels contained partially hemolysed blood. Perivascular area showed cellular reactions forming granulomatous lesions where as in case of *C. inscriptus* and *C. lentiginosus* hyperplasia of cortical cells was detected with constriction of sinusoidal space. Hepatic cells showed marked pycknosis of the nuclei and extremely vacuolated areas with honey-comb-like appearance in case of mice injected with the venom of these two species. In *C. inscriptus* widespread diffused hemorrhagic area could be noticed in the cortico-medullary junction in case of the mice injected with *C.
inscriptus venom. Hemorrhages were evident at the medullary region and the cortical region showed accumulation of edema fluid, whereas in case of C. inscriptus and C. zeylanicus occasional shrinkage of the glomerular tuft could be seen besides wider areas of hemorrhage throughout the kidney parenchyma. Blood vessels were severely congested with hemolysed blood. However, the cortex did not show any specific changes.

CONCLUSION
Present studies could explore and characterized the active agent responsible for toxicity, in order to reveal the biopharmacological properties of the Conus toxin. The gross behavioural symptoms and histopathological observations in the current investigation confirm the toxic effect of the venom on kidney, liver and brain. It is concluded that the toxic effects are due to multigorgan system failure. Histopathological study revealed accumulation of polymorphic nuclear cells with necrosis in brain, hemolysis in heart, occlusion with hemolysed blood in kidney and necrosis, vacuolation with pleomorphic nuclear material and hemolysis in liver. The data obtained from the present study will be helpful for a better assessment of the clinical manifestations produced by the venomous marine animals.

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