

Cytotoxicity and bioactivity of some marine animals

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Organic extracts of locally occurring animals such as sponge *Tethya* sp., hermit crab *Eupagurus* sp., cuttle fish *Sepia aculeata*, squid *Loligo duvaucelli*, starfish *Asterina lorioli* and flat fish *Cyanoglossus* sp. Were tested for fish toxicity, haemolytic activity and larvicidal activity. All extracts were toxic to fish resulting in death of fish accompanied by changes in gills and liver. Chloroform extract of sponge, methanol extract of hermit crab, ether fraction of acetone extract of *Loligo duvaucelli*, *Sepia aculeata* and *Cyanoglossus* sp. showed strong haemolytic activity. Chromatographic separation of extract of *Loligo* yielded a highly haemolytic white shining compound, (m.p. 145-146°C). Chloroform extract of starfish was strongly haemolytic than petroleum ether extract. Petroleum ether extract of starfish and sponge showed strong haemolytic activity.

The marine environment is characterised by physical and chemical properties that are markedly different from those of terrestrial environment. The marine environment comprises of complex ecosystems and many of these organisms are known to possess bioactive compounds as a common means of defense especially in sessile or slow moving benthic animals. Bioactive substances from marine organisms have been studied for several decades and many chemicals have pharmacological properties¹⁻³.

Groups of animals that have been found to produce bioactive natural products include bacteria, dinoflagellates, algae, coelenterates, sponges, bryozoans, soft bodied molluscs and echinoderms. Sponges are a good source material for a variety of bioactivity and structurally intriguing compounds including a number of antibiotics^{4,5}. Probably the most extensively studied animals among the marine invertebrates are echinoderms especially starfishes. Starfish extract is found toxic to fish, insects, causes liver damage⁶, antitumour activity⁷ and various pharmacological activities like cytolytic, analgesic, antiinflammatory and hypotensive⁸. Our earlier work on similar lines^{9,10} encouraged us to carry out further studies on locally available marine animals. The use of marine resources for pesticides, larvicides and ichthyotoxic is rare. With this objective in mind, we have been working on these aspects of various extracts of marine animals.

Materials and Methods

Fresh specimen of squid *Loligo duvaucelli*, cuttle fish *Sepia aculeata* (Mollusca, Cephalopoda) and flat fish *Cyanoglossus* sp. were collected from landing site, Versova along Mumbai coast. The specimens were macerated in acetone and the acetone extract was washed with solvent ether to obtain ether soluble fraction (L₁)(S₁). Starfish *Asterina lorioli*, sponge *Tethya* sp. and hermit crab *Eupagurus* sp. were collected from rocky shore near Juhu along Mumbai coast. The starfish and sponge were air dried and successively extracted in petroleum chloroform and methanol. *Eupagurus* sp. was directly extracted in methanol. Studies were carried out for fish toxicity, lethality and histopathological studies, haemolytic activity, larvicidal activity and chromatographic separation. Thin layer chromatography of various extracts mentioned above was carried out on commercially available TLC plates (Merk-mesh 250-400) using various solvent systems such as petroleum ether-ethyl acetate (6:4), benzene-methanol (1:1), petroleum ether-ethyl acetate-acetone (1:1:5).

Toxicity experiments were designed with common edible freshwater fish *Tilapia mossambica* (Peters) of average length 4.2 cm and 1.02 g body weight. Acclimated fish (temp. 27°C and O₂ 5 ppm) were placed in two jars (one as control and other as treated fish) containing different extracts of 1mg/ml dissolved in 250 ml of water. Experiments were terminated when the fish either showed normal consistent behaviour or died due to toxic effect. Time of death and histopathological changes were recorded. Tissues

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such as gill, liver, intestine were removed immediately after death of experimental fish, fixed in bouins fluid and processed for histopathological studies according to standard microtechnique procedure¹³. The tissue sections were stained with haematoxylin and eosin.

For biological activity studies haemolytic assay was performed according to method of Zvi Paster¹¹. Human RBC's suspended in saline were incubated at 37°C with various extracts for 1 hour, centrifuged (REMI R8C) and the absorbance of supernatant was noted at 530 nm colorimetrically (ERMA AE-11N). Lysis of erythrocytes in distilled water was considered as 100% lysis. One HD unit (haemolytic dose) was determined as that quantity of toxin that will lyse 50% of RBC's in the medium. For larvicidal activity laboratory reared IIIrd and IVth instar larvae of mosquito *Culex pipens fatigans* were used and activity determined according to method recommended by WHO¹². Observations for survival of larvae were made and mortality recorded after 24 hours.

Results and Discussion

In the present study we investigated extracts and fractions of locally available marine animals such as sponge *Tethya* sp., hermit crab *Eupagurus* sp., squid *Loligo duvaucelli*, cuttle fish *Sepia aculeata*, starfish *Asterina lorioli* and flat fish *Cyanoglossus* sp. mainly for haemolytic and larvicidal activities after initial toxicological studies.

Tilapia fish is a good model for determination of toxic effects of compounds of interest. In this study only the acute toxicity of various extracts was determined at a concentration of 1mg/ml, the results of which are summarized in Table 1. The ether fraction of acetone extract of *Cyanoglossus* showed maximum toxicity. The fish on exposure to test extracts showed various behavioural changes which were classified and graded (Table 2).

The larvicidal activity of various extracts is shown in Table 3. The petroleum ether extract of both sponge *Tethya* sp. and starfish *Asterina lorioli* were found to be most active against mosquito larvae. These results suggest that these extracts can provide new compounds for developing potential larvicides. It is obvious from the data that the extracts showing strong haemolytic activity did not show significant larvicidal activity.

Further evidence of toxicity signs is provided with histological data (Table 4). Gills show

pronounced changes being respiratory organs and are directly exposed to the biotoxin. Gills show either bulging or necrosis with destruction of primary and secondary lamellae (Fig. 1). The liver also shows effect of test extracts, in that cells show vacuolation of cytoplasm and nucleus. There is also severe necrosis in some of the treated tissues with total destruction of hepatocytes (Fig. 2). However the intestinal tissue does not show any effect of the biotoxins, indicating that the path of the toxin is gills-blood vessels-liver.

Table 1—Toxicity (lethality of different extracts (1 mg/ml) on fish *Tilapia mossambica*

Animal	Extract	Time for death h/min
<i>Tethya</i>	Petroleum ether	1.50
	Chloroform	—
<i>Eupagurus</i>	Methanol	3.45
<i>Loligo</i>	Ether	1.15
<i>Sepia</i>	Ether	1.25
<i>Cyanoglossus</i>	Ether	0.56
<i>Asterina</i>	Petroleum ether	1.35
	Chloroform	1.20

Table 2—Behavioral changes observed in fish *Tilapia mossambica* during acute toxicity test

Stage I	:	Initial signs
		a) Increase in ventilatory frequency
		b) Increase in activity
Stage II	:	Secondary signs
		a) Leaning to one side
		b) Loss of swimming activity
Stage III	:	Advanced signs
		a) Leaning on bottom or against aquarium side.
		b) Start of sporadic uncontrollable swimming with non directional bursts

Table 3—Larvicidal activity in terms of percentage mortality (2 mg/ml extract was added)

Animal	Extract	Mortality (%)
<i>Tethya</i>	Petroleum ether	90
	Chloroform	50
<i>Eupagurus</i>	Methanol	1
<i>Loligo</i>	Ether	Nil
<i>Sepia</i>	Ether	Nil
<i>Cyanoglossus</i>	Ether	30
<i>Asterina</i>	Petroleum ether	100
	Chloroform	Nil

Haemolytic activity as a measure of cytotoxicity has shown significant results. The petroleum ether extract test on sponge *Tethya* sp. showed less haemolytic activity but chloroform extract has maximum lytic activity at $HD_{50} < 2$ mg/ml (Fig. 3A), indicating the presence of toxin in the chloroform extract. The methanol extract of hermit crab *Eupagurus* shows haemolytic activity at HD_{50} 4.6 mg/ml (Fig. 3B). The ether fractions of *Loligo duvaucelli* and *Sepia aculeata* show significant haemolytic activity at HD_{50} 3.1 and 2.85 mg/ml concentration respectively (Fig. 3D). Chloroform extract of starfish *Asterina lorioli* is more haemolytic than the petroleum ether extract with HD_{50} values 3.4 mg/ml and 7.7 mg/ml respectively (Fig. 3C). Methanol extract of *Cyanoglossus* did

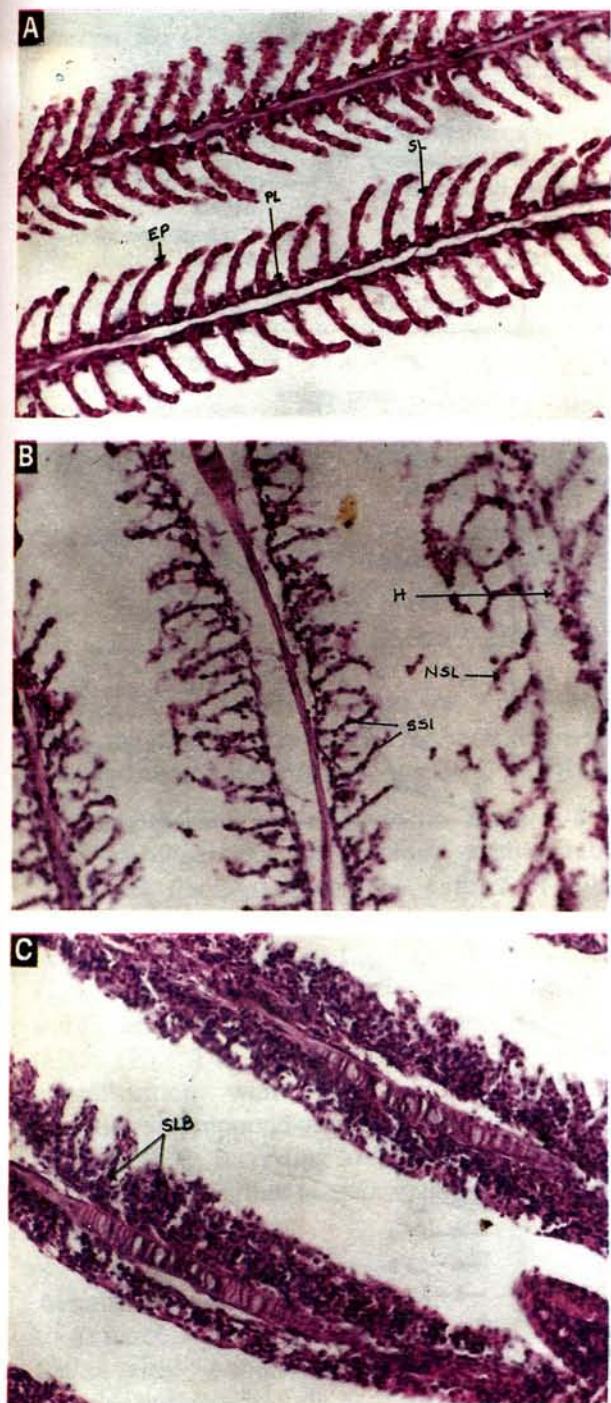


Fig. 1—(A) Section of normal gill of *Tilapia mossambica*, (B) Section of gill showing necrosis of gill lamellae (NSL) haemocytes (H). (C) Section of gill showing bulges in secondary lamellae (SLB) PL—primary lamellae, EP—epithelial cell, SL—secondary lamellae.

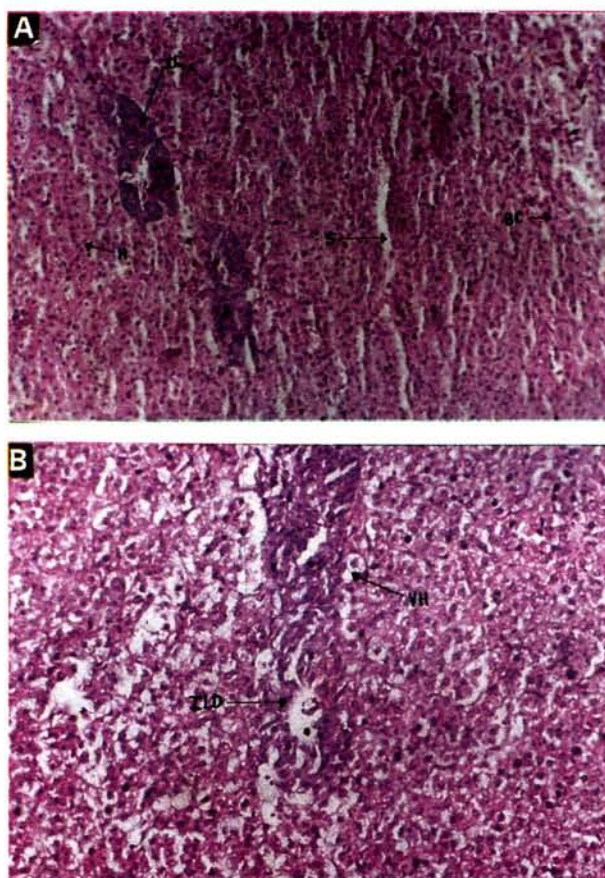


Fig. 2—(A) Section of normal liver of *Tilapia mossambica*, (B) Section of treated liver of *Tilapia mossambica* HP—hepatocytes, IL—Islets of Langerhans, S—sinusoids, BC—blood capillaries.

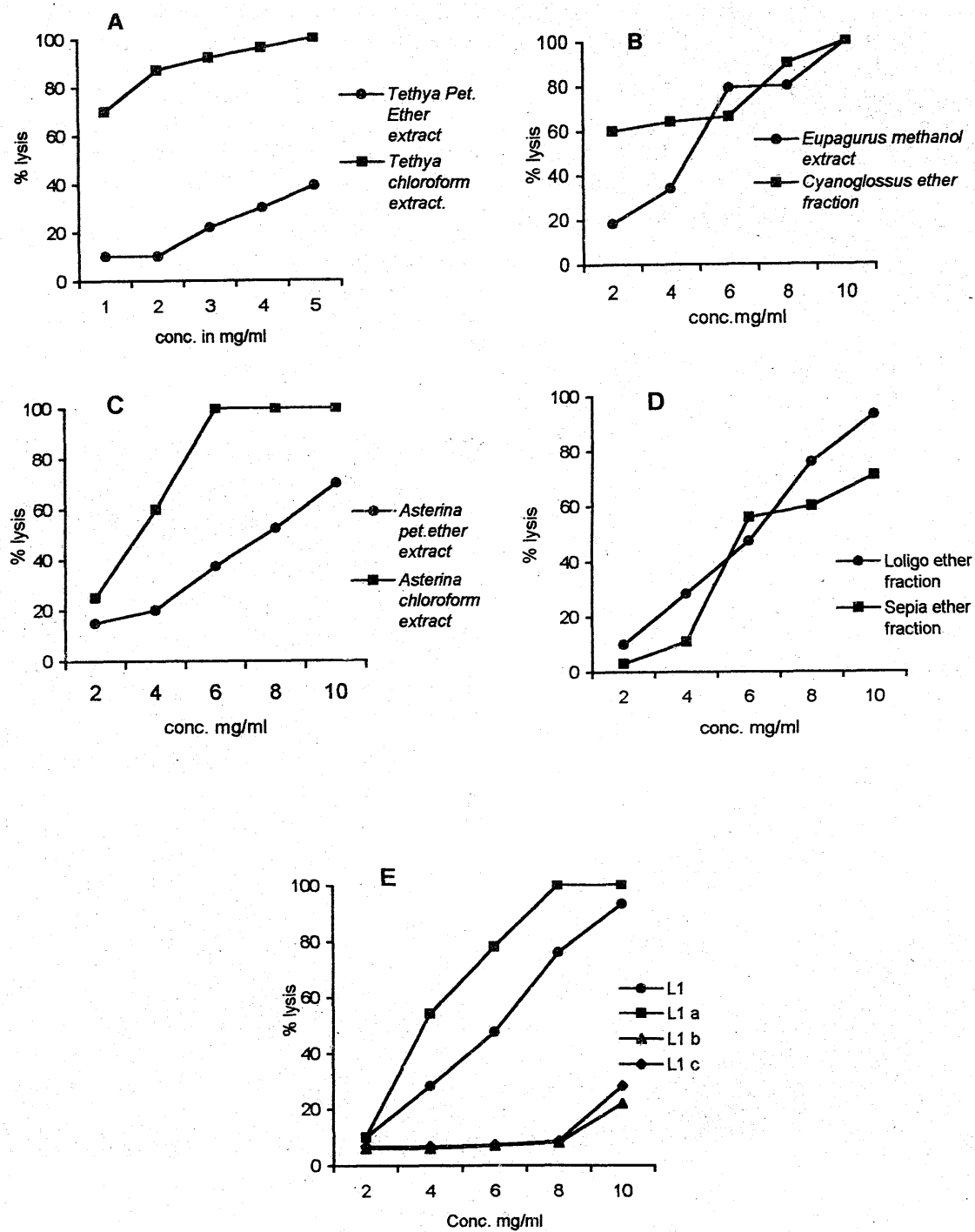


Fig. 3—(A) Comparative haemolytic activity of extracts of sponge *Tethya*, (B) haemolytic activity of hermit crab *Eupagurus* and *Cyanoglossus* extracts, (C) haemolytic activity of squid *Loligo* and cuttle fish *Sepia* extracts, (D) comparative haemolytic activity of extracts of starfish *Asterina lorioli*, (E) comparative percentage haemolysis of subfractions (L_{1a}, L_{1b} and L_{1c}) of ether fraction of acetone extract of *Loligo duvaucellii* (L₁)

Table 4—Histopathological changes observed in gill and liver of fish *Tilapia mossambica* when exposed to extracts from marine animals

Extracts	Gill	Liver
Petroleum ether of <i>Tethya</i> sp.	Destruction of primary lamellae and splitting of secondary lamellae	Coagulative necrosis of hepatocytes. Blood sinuses and capillaries not disturbed, RBC's absent.
Methanol of <i>Eupagurus</i> sp.	Tip of primary lamellae swollen filled with blood cells. Secondary lamella necrosed and vacuolation.	Cells vacuolated hypochromatic nucleus, blood sinuses intact, endothelial lining disturbed.
Ether of <i>Loligo duvaueilli</i>	Swollen structure of secondary lamellae lost and proximal epithelial cell ruptured.	Vacuolation of hepatocytes with pycnotic nucleus and destruction of blood cells.
Ether of <i>Sepia aculeata</i>	Semilunar structure of secondary lamellae lost and proximal epithelial cell ruptured.	Disturbed hepatic cords. Hepatocytes separated. Vacuolated nucleus. RBC's absent.
Petroleum ether of <i>Asterina lorioli</i>	Destruction of primary lamellae. Haematocytes fill primary lamellae. Cells of secondary lamellae necrosed and show splitting.	Severe necrosis of hepatocytes with vacuolation. Nucleus intact.
Chloroform of <i>Asterina lorioli</i>	Primary lamellae swollen. Total destruction of secondary lamellae.	Hepatocytes showing necrosis, nucleus not changed. Destruction of capillaries.
Ether of <i>Cyanoglossus</i>	Swelling of primary lamellae Secondary lamellae broken in few places.	Cords are disturbed. Cells vacuolated. Enlarged, vacuolated nucleus-pycnotic.

not exhibit any haemolytic activity. However, the ether soluble fraction of its acetone extract is strongly haemolytic at $HD_{50} < 2 \text{ mg/ml}$ (Fig. 3B).

Attempts were also made to isolate active fractions by carrying out chromatographic studies. The ether fraction of acetone extract of *Loligo duvaueilli* was applied on silica gel column to obtain 3 subfraction (L_{1a} , L_{1b} , L_{1c}). The subfraction L_{1a} showed strong haemolytic activity while L_{1b} and L_{1c} did not show significant haemolytic activity (Fig. 3E). The subfraction L_{1a} on repeated crystallization with ethyl alcohol yielded a crystalline compound as white shiny flakes, m.p. 145°C – 146°C , according to Libbermann-Bruchardt test for sterol. Further characterization of this sterol is presently underway.

Acknowledgement

Authors wish to thank Dr. A.K. Modi (Modi Pathological Laboratory) for providing blood samples during the study.

References

- McCaffrey E E & Endem R, *Mar Biol*, 89 (1985) pp 1.
- Kamat S Y, Wahidulla S, D'Souza L, *et. al. Bot Mar*, 35 (1992) 161.
- Schmitz F J, Gunasekara S R & Yamanichili G, *J Chem. Soc*, 106 (1984) 7251.
- Nigrelli R S, Jakowska S & Carlent I, *Zoologica (N.Y.)*, 44 (1959) 173.
- Sarama N, Rajaneyulu & Rao C B S, *Indian J. Chem.*, 26 (1987) 189.
- Kazuo S, Shinsuke Y, Hideaki Y, Takeaki K & Kenjiro K, *Toxicon*, 28 (1990) 469.
- Ruggieri G D & Nigrelli R F, in *Bioactive compounds from the sea*, edited by Humm H J & Lane C S (New York: Marcel Dekker) (1974) 183.
- Owells R J, Owells R C, Coorg M A & Klien D, *Toxicon*, 11 (1973) 319.
- Indap M M, Thakur N L, Zarapkar S S & Shivalkar S A, *Indian J. Exp. Biol*, 34 (1996) 588.
- Indap M A & Ambaye R Y, *Indian Drugs*, 29 (1991) 43.
- Zvi Paster, *Marine pharmacognosy*, (Academic Press, New York) 1973, 248.
- Chavan S R, Nikam S T, *Bull Haffkine Institute*, 10 (1982) 21.
- Weesner F M, *General zoological microtechniques*, (The William and Wilkins Company, Baltimore (1968) 163.