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Studies on Haemolytic Properties of Puffer Fishes from South East Coast of India

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ABSTRACT

Marine fishes play an important role for health care. The objectives of the present study was to evaluate the haemolytic activity of crude extracts of six puffer fishes *Cyclichthys orbicularis, Diodon holocanthus, Canthigaster solandri, Arthron hispidus, A. inermis* and *Lagocephalua inermis* collected from Parangipettai, Tamil Nadu, South East Coast of India. The haemolytic activity was tested against red blood cells (RBCs) of chicken, goat and human blood. The haemolytic activity was high in chicken blood (128HU) against of *Cyclichthys orbicularis* and *A. inermis* and minimum (16HU) in *Lagocephalua inermis*. In goat erythrocytes the highest haemolytic activity (256HU) against *Arthron hispidus* and minimum (16HU) against *A. inermis* and *Lagocephalua inermis* were observed. In human erythrocytes the maximum haemolytic activity of 32HU against *Cyclichthys orbicularis, Arthron hispidus* were recorded. In blood agar plate assay the highest zone of inhibition of 6.4±0.9 and 6.3±0.1 mm were observed in *A. inermis* against chicken erythrocytes and *Arthron hispidus* against goat erythrocytes respectively. The results strongly suggest that, the Marine puffer fish extracts showed good cytolytic properties against blood RBCs.

***Keywords*:** South East Coast of India; haemolytic activity; health care; marine fishes

1. INTRODUCTION

The world's oceans, covering more than 70% of the earth surface represent enormous resources for the discovery of potential chemotherapeutic agents that may be useful for finding drugs with greater efficacy and specificity for the treatment of many human diseases (wright et al., 1996 and Faulkner, 2001). The study of natural products that exhibit biological activity derived from plants and animals has long been known for their significant biomedical value and the crude products isolated from marine organisms have served as a source of many drugs. marine organisms not only elaborate pharmaceutically useful compounds but also produce toxic substances and fabricate some of the most cytotoxic compounds ever discovered, but the yields of these compounds are invariably so small that natural sources are unlikely to provide enough material for drug development studies.

Fishes are one of the diverse sources of natural products and bioactive compounds with over 40,000 known species. They combat infections caused by viruses, bacteria, fungi and parasites that are similar to those of humans and other vertebrates. Many species of marine

fish have been reported as ithyocrinotoxic (halstead, 1978), releasing in to the water toxic secretions. The chemistry of these secretions was studied in only a few representatives of "soap" serranidae, tetrodontidae (puffer)" toad" (batrachoitididae), "flat" (solidae) and "trunk" (box) ostraciidae fishes, have been isolated in "pure" form and tested for biological activity.

Puffer fishes are notorious for their potent toxin, making the fish inedible unless prepared by skilled fugu chefs. The toxin these fish possess is a tetradotoxin (ttx),named for its presence in fish of the order tetraodontiformes including puffer fishes and porcupine fishes and is then concentrated in the skin and liver of the fish. The toxin is produced by several bacteria species including mycobacterium *Arabino galatanolyticum*, *Serratia marcescens*, *Vibrio alginolyticus* and *Bacillus spp*. (Yu et al., 2001 and Wu et al., 2005). TTX-producing bacteria from *Arothron hispidus* also hold promise for the development of effective antitumor compounds (Bragadeeswaran et al., 2010).

Most of puffer fish toxins (ithyocrinotoxins) causes hemolysis of erythrocytes. Hemolysis is the breakdown of red blood cells and in the final stage of breakdown haemoglobin is released from the red cells (Simonsen et al., 2008). Hemolysis in small amount is a normal body process. About 0.8 to 10% of all red cells in the body are hemolysed each day. It is usually balanced by red cell production in the marrow of bones. But sometimes, so many cells breakdown that marrow production is insufficient and anaemia may result. Chemical poison may cause excessive hemolysis. Many biotoxins are known to cause hemolysis of RBC. Haemolytic activity has been observed in many of the tissue products of aquatic organism including fish, molluscs, algae etc. Most cytotoxic have considerable potential as anticancer and antiviral agents (Shier, 1988). The potential of puffer fishes as a source of biologically active products is largely unexplored. Hence a broad based screening of puffer fishes for bioactive compounds is necessary.

2. SAMPLE COLLECTION AND PRESERVATION

The marine puffer fishes (*Cyclichthys orbicularis, Diodon holocanthus, Canthigaster solandri, Arthron hispidus, A. inermis* and *Lagocephalua inermis*) were collected from the lading center (Lat. 11º30.47N; Long. 079º47. 02E) of Annangkovil, Parangipettai, Tamilnadu, South east coast of India during summer. Collected poisonous puffer fishes were transported to the laboratory carefully.

3. COLLECTION OF CRUDE SAMPLE

Fresh tissue samples were collected from the clearly washed specimens and extracted with methanol at 37 ºC for 3 days. The extracts were filtered through Whatman No. 1 filter paper and the solvents were concentrated using by rotary evaporator (VC100A Lark Rotavapor at 30 ºC) under reduced pressure. The yield of dark brown gummy mass was stored at 4 ºC for further analysis.

4. HEMOLYSIS STUDY

4. 1. Preparation of erythrocyte suspension

Fresh chicken blood has been collected from the nearby slaughter house in Parangipettai and was added with EDTA solution 2.7 g in 100 mL of distilled water as anticoagulant at 5 % of the volume of blood. The blood was centrifuged at 5000 rpm for 7 minutes at 4 °C along with normal saline about double the quantity of blood. The supernatant was discarded. 1 mL of the packed RBC thus obtained has been resuspended in normal saline to obtain a 1% RBC suspension

4. 2. Haemolytic assay

The assay was carried out according to Pani Prasad and Venkateshvaran (1997) in "V" shaped Laxbro microtitre plates. The lyophilized toxin and the lethal fractions were assayed. The concentration of the toxin was 5 mg/ mL. One row of well was used for only one toxin fraction. Initially 100 µL of normal saline was added to each well. Then 100 µL of the first toxin fraction was added to the first well and was thoroughly mixed. From this 100 µL was transferred to the next well and this process was repeated upto the last well from which 100 µL of the dilution was discarded. Then 100 µL of the prepared erythrocyte suspension was added to each well. Simultaneously a negative control has been kept by mixing 100 µL of normal saline and 100 µL of 1% RBC suspension. The plates were incubated for 2 hours at room temperature and the results were taken Formation of a fine "Button cell" with regular margin indicates the negative reaction. A uniform red colored suspension of the lysed RBC indicates the positive result. Haemolytic activity was expressed as Haemolytic Unit (HU). 1 HU being defined as the amount of protein required to cause 50 % hemolysis or the reciprocal of the highest dilution of the toxin in which a haemolytic pattern is obtained.

4. 3. Haemolytic assay on blood agar plate

The haemolytic activity was assayed using blood agar plates by following the method of Lemes-Marques and Yano (2004). Chicken and goat blood agar plates were prepared by adding 5 ml of blood to 95 ml of sterile blood agar aseptically, with the result poured immediately onto the Petri dishes. After solidification, wells were cut into the agar plate-using a corkscrew borer (8 mm diameter). Wells were loaded with 50 µl (1 mg/ml) of samples. The plates were observed for hemolysis after overnight incubation at room temperature

5. DISCUSSION AND CONCLUSIONS

All blood groups showed good activity. The crude methanolic extract of marine puffer fishes *Cyclichthys orbicularis, Diodon holocanthus, Canthigaster solandri, Arthron hispidus, A. inermis* and *Lagocephalua inermis* produced pronounced haemolytic activity on chicken, goat and human erythrocytes. Haemolytic factors were present in all the six puffer fishes, but differed considerably depending on the type of blood used. Goat blood the most vulnerable hemolysis was observed from the crude extract *Arthron hispidus* showed the maximum haemolytic unit (256 HU) and minimum of 4HU was recorded in human blood. Hemolysis of human and sheep red blood cells has been studied by Al-Hassan et al. (1982).

In chicken blood cell the highest haemolytic activity was observed at crude extract of *Cyclichthys orbicularis, A. Inermis* (128HU) and minimum in 16HU at *Lagocephalua inermis*. The earlier work Chicken blood showed high haemolytic activity on the skin tissue

extracts when compared to liver, muscle extract as same as reported by Bragadeeswaran et al., 2010 has studied the haemolytic potential of tetrodotoxin producing bacteria in A. hispidus. Whereas the human blood showed moderate activity. In human blood cell the maximum haemolytic activity was observed at *Cyclichthys orbicularis* and *Arthron hispidus* (32HU) and minimum in 4HU at *Diodon holocanthus*. The mucus secretion of fresh water eel *M.armatus* has proteinaceous substance which shows potent bioactivity (haemolytic)when mixed blood cells of sheep and cow(Venkatachalam Uthayakumar et al., 2012).

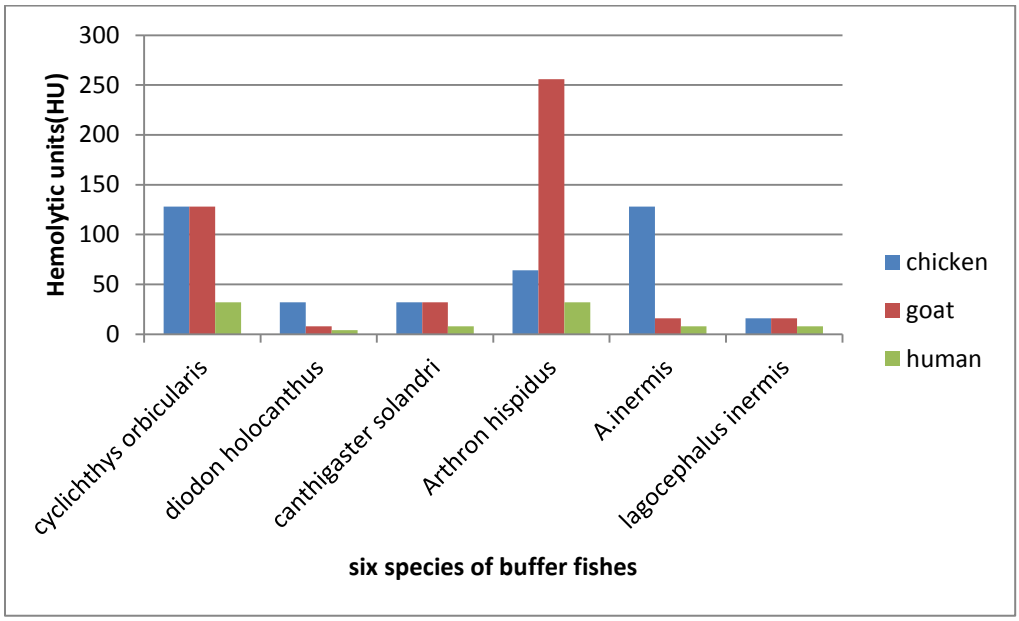


Fig. 1. Haemolytic activity of puffer fish extract against chicken, goat and human.

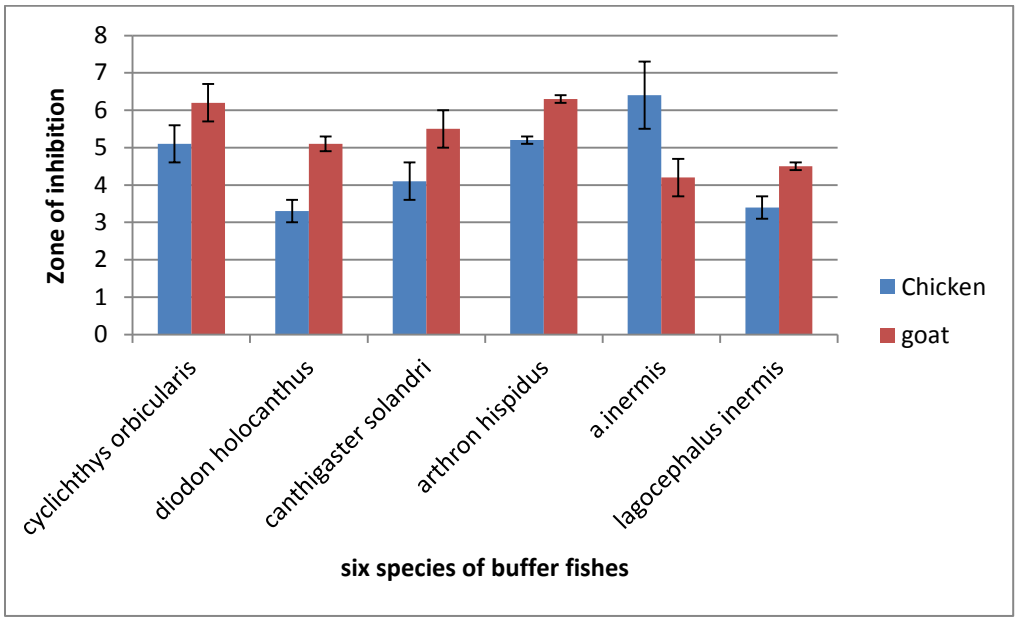


Fig. 2. Haemolytic activity of blood agar plate against chicken and goat.

Table 1. Haemolytic activity of puffer fish extract against chicken.

Serial no	Crude methanolic extract	Type of blood grouping	Sample concentration (200µg/ml)	Total hemolysis up to dilution	Haemolytic titre value (HU)
1	*Cyclichthys orbicularis*	chicken	200µg/ml	7	128
2	*Diodon holocanthus*	chicken	200µg/ml	5	32
3	*Canthigaster solandri*	chicken	200µg/ml	5	32
4	*Arthron hispidus*	chicken	200µg/ml	6	64
5	*A. inermis*	chicken	200µg/ml	7	128
6	*Lagocephalua inermis*	chicken	200µg/ml	4	16

Table 2. Haemolytic activity of puffer fish extract against goat.

Serial no	Crude methanolic extract	Type of blood grouping	Sample concentration (200µg/ml)	Total hemolysis up to dilution	Haemolytic titre value (HU)
1	*Cyclichthys orbicularis*	goat	200µg/ml	7	128
2	*Diodon holocanthus*	goat	200µg/ml	3	8
3	*Canthigaster solandri*	goat	200µg/ml	5	32
4	*Arthron hispidus*	goat	200µg/ml	8	256
5	*A. inermis*	goat	200µg/ml	4	16
6	*Lagocephalua inermis*	goat	200µg/ml	4	16

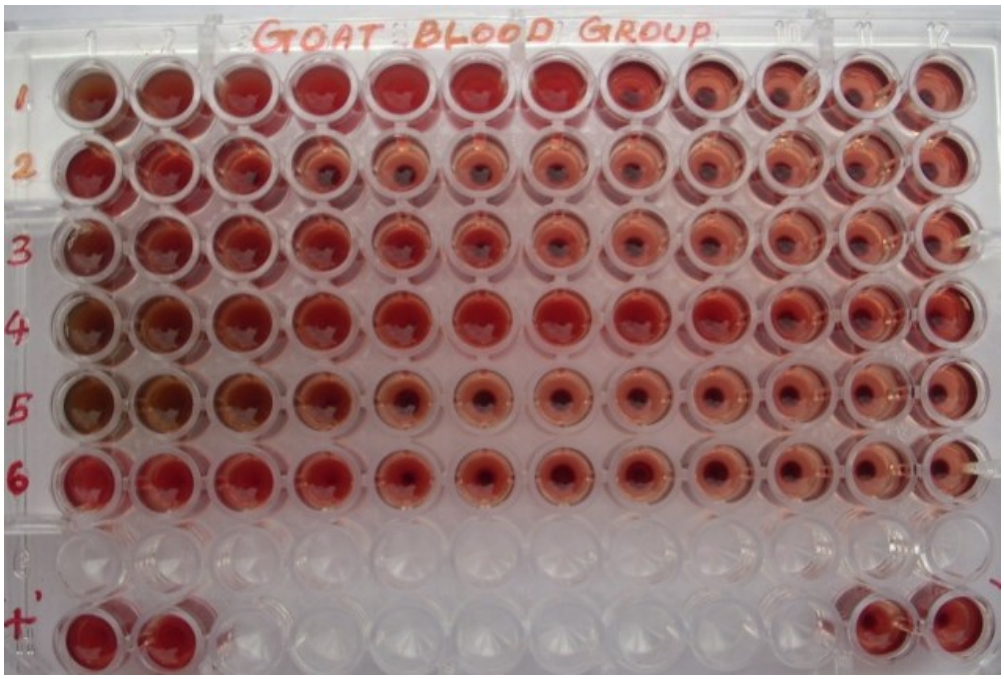
Table 3. Haemolytic activity of puffer fish extract against Human.

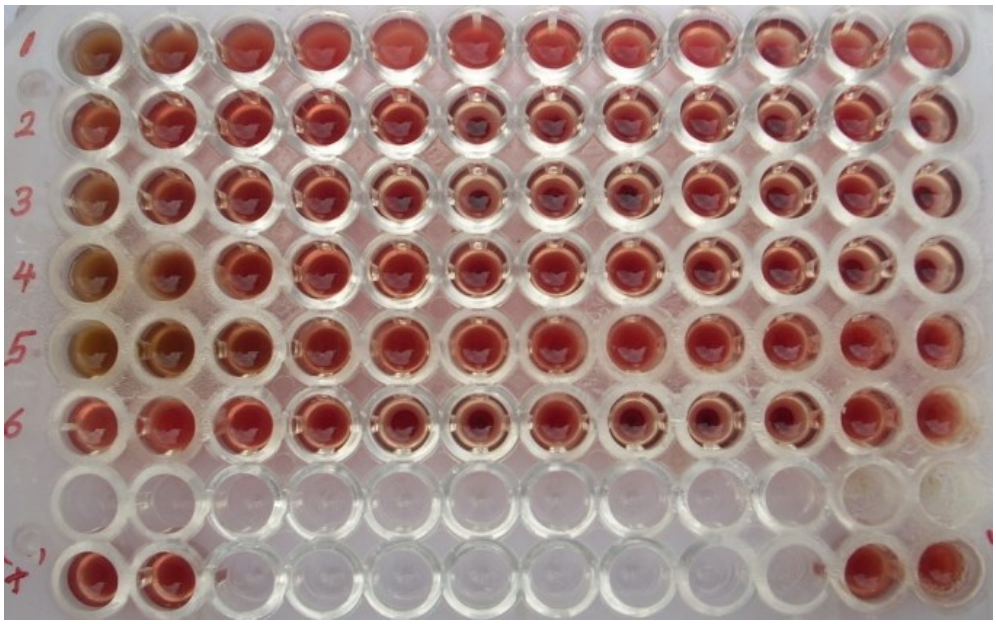
Serial no	Crude methanolic extract	Type of blood grouping	Sample concentration (200µg/ml)	Total hemolysis up to dilution	Haemolytic titre value (HU)
1	*Cyclichthys orbicularis*	Human	200µg/ml	5	32
2	*Diodon holocanthus*	Human	200µg/ml	2	4
3	*Canthigaster solandri*	Human	200µg/ml	3	8

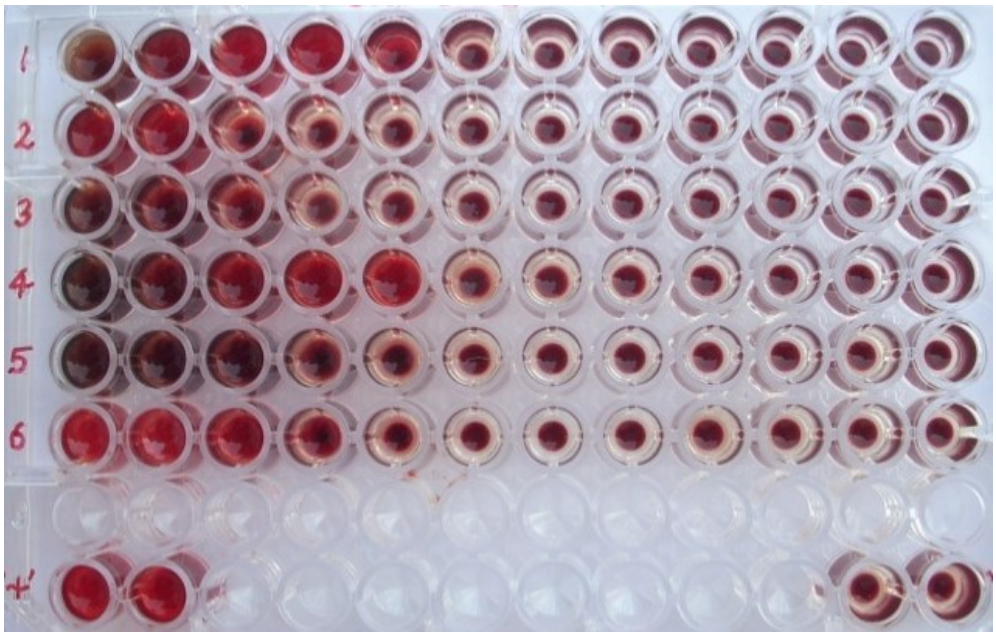
4	*Arthron hispidus*	Human	200µg/ml	5	32
5	*A. inermis*	Human	200µg/ml	3	8
6	*Lagocephalua inermis*	Human	200µg/ml	3	8

Table 4. Haemolytic activity of blood agar plate against chicken and goat.

Serial No	Sample	Zone of Inhibition(mm)	
		chicken	Goat
1	*Cyclichthys orbicularis*	5.1±0.5	6.2±0.5
2	*Diodon holocanthus*	3.3±0.3	5.1±0.2
3	*Canthigaster solandri*	4.1±0.5	5.5±0.5
4	*Arthron hispidus*	5.2±0.1	6.3±0.1
5	*A. inermis*	6.4±0.9	4.2±0.5
6	*Lagocephalua inermis*	3.4±0.3	4.5±0.1







The capacity of crude extract to lyses red blood cells was found by performing haemolytic assay on microtitre plates and blood agar plates. In the present study, more haemolysis has occurred in goat and chicken blood. Soluble and in soluble proteins from mastacembalus armatus have tendancy to cause haemolytic effects (venkatachalam uthyakumar et al., 2012). It is evident from the present study that the puffer fish species have potent haemolytic against various red blood cells. The maximum zone of inhibition in blood agar plate on (6.4 ±0.9 mm) against chicken and minimum in (3.3 ±0.3 mm). In goat blood agar plate produced highest in (6.3 ±0.1 mm) and lowest in (4.2 ±0.5 mm).

Similarly The toxin from soft coral *Sarcophyton trocheliophorum* shows significant inhibitory effect on blood agar plate (Karthikayalu et al*.,* 2010) and fish epidermal mucous shows significant haemolytic effect (Bragadeeswaran et al., 2011). The results clearly indicate the toxins present in the fish are having bioactive compounds that may be used for therapeutic needs.

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