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Bioactive Potential of Puffer Fish Arothron Stellatus collected from South East Coast of India

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Abstract:
Puffer fish, *Arothron stellatus* collected from Kasimedu fish landing station, Chennai was studied for its biological activity. Toxin was extracted from the skin of the fish using 0.1% acetic acid. The crude skin extract has shown inhibition activity against the bacterial and fungal strains. It has shown the maximum activity against *E. coli* and *S. aureus* and minimum activity against *K. pneumoniae,* antifungal activity was observed maximum against *A. niger* and *A. flavus* and minimum activity against *T. viridae.* Cytotoxicity of skin extract of fish was checked against HeLa cell line by MTT assay and the IC_{50} value were found to be 64.86 µg/ml. The results of the present study reveals puffer fish *A. stellatus* has bioactive compound which can be utilized for further development as drug.

Keywords: Puffer fish, *A. stellatus,* Cytotoxicity, Antimicrobial activity.

Introduction

Marine ecosystem covers nearly 70% of the earth's surface. Most of the marine organisms live in a hostile environment, having developed a well defence mechanism for their survival (1). Marine ecosystem has drawn attention of many researchers due to its rich and renewable source (2). The bio resources present in the marine ecosystem have potent biomolecules which includes many natural organic compounds. These compounds are reported to have biological activities like anti- tumor, anti- viral, analgesic, etc (3) Marine fish are also of high nutritive value. Proteins from fish have shown biological activities like anti- bacterial, anti- inflammatory, anti-oxidant, etc. (4). Fish live in intimate contact with an environment containing pathogenic organisms. The slow adaptive immune response of fish makes innate immunity, which is fast acting and temperature independent predominant system of fish host defence (5). The defence includes many elements such as antimicrobial peptides, lipids and polypeptides (6). Puffer fish Arothron immaculatus belongs to the family Tetraodontidae commonly found in the tropical regions, containing tetrodotoxin (TTX). Tetrodotoxin, a well-known marine neurotoxin due to its unique chemical structure and specific sodium channel blocking action of excitable membranes mainly causes respiratory paralysis and intoxication (7). During the World War II, the crude puffer fish extracts have been used for treating migraines and menstrual cramps (8). Poisoning due to TTX toxicity is recorded form 17th century, however, from the past few years, TTX is studied for its potential for identification, isolation and characterization of voltage-gated sodium

channels. In 1992, Tosteson demonstrated that TTX has a great ability to bind to the trans-membrane glycoprotein forming the Na^+ channel which resulted in blocking it. It acts on sodium channel without affecting the permeability of the potassium ion channel. This specific binding ability of TTX has gained importance in biomedical research. Several reports have been published concerning the effect of TTX on cells of different size, type and shape. A group of scientists performed clinical trials in Canada and China using tetrodotoxin as an analgesic which reduces the intense pain caused by advanced stage of cancer in patients. The clinical trials indicated that a formulation of TTX, Tetrodin was safe when given in very small doses (9). It is also reported to have potentials to alleviate acute heroin withdrawal syndrome in addicts with few side effects (10). The present study was carried to screen the antimicrobial, haemolytic and cytotoxic activity of crude extract of the puffer fish *A. stellatus* in order to study the other potential activities.

Materials and Methods

Sample collection:

The puffer fish, *Arothron Stellatus* (Lacepede 1958) (figure. 1) was collected from the Kasimedu fish landing station at Chennai coast 13°7'N 80°17'E, east coast of India. Fish specimens were identified based on the appearance and external morphology by Dr. S. S. Khora, Sr. Professor, SBST, VIT University. Samples were transferred to the laboratory in dry ice and stored at -20 ºC until use.

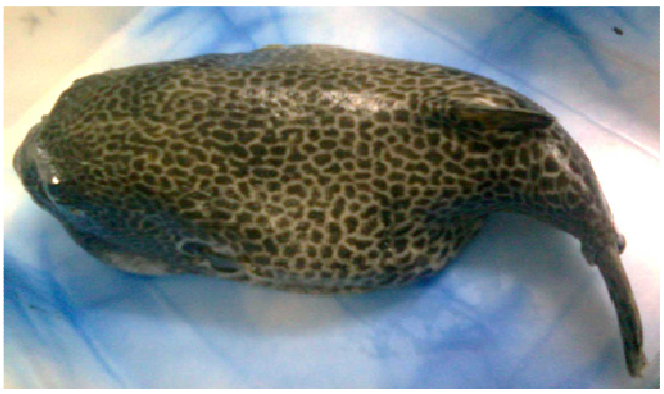

Fig 1: *Arothron stellatus* collected from Kasimedu fish landing station, Chennai coast.

Extraction of crude extract

After thawing the fish was carefully excised for its skin. Ten grams of skin was homogenized with 0.1% acetic acid and boiled for 10 minutes followed by centrifugation at 5000 rpm for 15 minutes. The above step was carried out thrice to extract the toxin (Khora, 1991). Supernatant obtained was concentrated using rotary evaporator and stored at -20º C for further use. (11).

Tested organisms

Bacterial species such as *E. coli, S. aureus, B. cereus, B. subtilis, K. pneumoniae, P. vulgaris, and P. vulgaris* were maintained in Luria Bertani broth. Fungal species such as A. *niger, A. flavus, A. fumigatus, C. albicans, T. viridae and T. rubrum* were maintained in Potato dextrose broth.

Antimicrobial activity

Antimicrobial activity of liver tissue extract was performed using the disc diffusion method according to Kirby with little modifications (12). Sterile disc of 8mm in diameter were impregnated with 30µl of tissue extract kept on the Muller Hinton agar plates which were previously swabbed and incubated at 37º C for 24 hrs and 72 hrs for bacterial and fungal cultures respectively. The diameter of the zone around the discs was measured and expressed in mm. Ampicillin and

fluconazole were used as a positive control for bacterial and fungal organisms.

Haemolytic activity

Haemolytic activity of the liver tissue extract was carried out using chicken blood collected from slaughter house, Vellore. The chicken blood was freshly collected. It was centrifuged, erythrocytes were collected and washed twice in sterile saline solution, centrifuged at 1500rpm for 5 min and the erythrocyte pellet was collected. Pellet was diluted with 1:9 (v/v) of saline solution and the crude extract with the various concentrations from (200, 400, 600,800 and 1000) µg/ml was added. 10% triton X and distilled water served as a positive and negative control. Blank were prepared without addition of blood. The mixture was vortexed and incubated at 37ºC for 1 hr, centrifuged at 8000 rpm for 10 min. The supernatant was collected and measured using UV spectrophotometer at 540 nm. Experiments were done in triplicates and the concentrations of the extract inducing 50% hemolysis were calculated and represented graphically (13)

The Percentage of hemolysis was calculated using the following formula,

$$\frac{Abs.\ of\ sample - Abs.\ of\ blank}{Abs.\ of\ positive\ control} \times 100$$

Cytotoxic activity of Skin tissue extract against HeLa cell line:

Cytotoxic activity of the skin tissue extract of *A. stellatus* was screened against the HeLa cell lines using the MTT assay (14). 1×10^6 Hela cell line in 100 µL DMEM medium with 10% FBS per well were plated in a 96 wells plate and incubated overnight at 37ºC in 5% CO_2 incubator. 1.5-100 µg / ml of the test samples were prepared in DMEM without FBS. 100 µL of DMEM was used as negative control and 5 µg / mL Doxorubicin was added as positive control. Wells without any cells are used as blank. The plates were gently shaken and incubated at 37ºC in 5% CO2 for 48 hrs. 20 µL of 5mg / mL MTT in PBS was added to each well and incubated at 37ºC in 5% CO2 for 4 hours. Then the medium was aspirated out and 200 µL of dimethylsulfoxide (DMSO) added to each well. The optical density of each well was measured using microplate reader at 505 nm. Then, the percentage of inhibition of cell growth is calculated as follows:

Percentage of inhibition = 100 – (Sample / Control) x 100

Results

The antibacterial activity of the crude extracts of puffer fish *A. Stellatus* were tested against the bacterial strains. The results of the inhibition activity of the crude extract of *A. stellatus* as shown in the table 1 as compared with the standard ampicillin. The maximum activity of the extract was shown against the *E. coli, S. aureus* and minimum activity against *K. pneumoniae, p. aeuroginosa* as shown in the fig 1.

Table 1: Antibacterial activity of *A. stellatus*

Bacterial strains	Crude extract (Zone of inhibition in mm)	STD – Ampicilin (Zone of inhibition in mm)
E. coli	11.32 ± 0.23	12.27 ± 0.34
S. aureus	11.10 ± 0.37	12.34 ± 0.43
P. vulgaris	10.38 ± 0.27	11.21 ± 0.31
B. cereus	10.21 ± 0.13	11.26 ± 0.35
K. pneumoniae	8.20 ± 0.43	10.12 ± 0.28
P. aeuroginosa	9.13 ± 0.38	10.21 ± 0.20

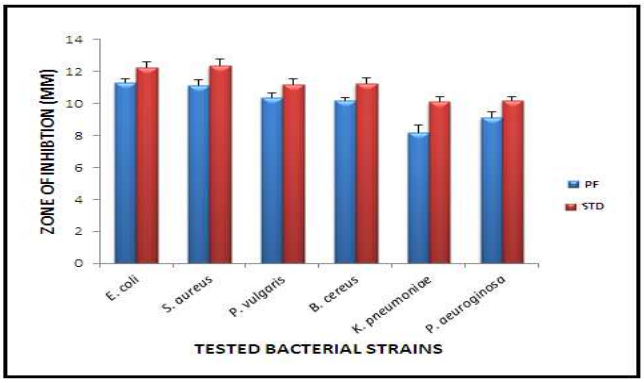

Fig 2: Antibacterial activity of *A. stellatus*

The antifungal activity of the crude extracts of puffer fish *A. Stellatus* were tested against the fungal strains. The results of the inhibition activity of the crude extract of *A. stellatus* as shown in the table 2 as compared with the standard fluconazole. The maximum activity of the extract was shown against the *A. flavus, A. fumigatus* and minimum activity against *T. viridae* and *T. rubrum* as shown in the fig 2.

Table 2: Antifungal activity of *A. stellatus*

Fungal strains	Crude extract (Zone of inhibition in mm)	STD – Fluconazole (Zone of inhibition in mm)
A. niger	*10.02 ± 0.14*	*11.23 ± 0.24*
A. flavus	*10.45 ± 0.21*	*11.43 ± 0.43*
A. fumigatus	*10.18 ± 0.17*	*11.01 ± 0.21*
C. albicans	*9.52 ± 0.11*	*11.16 ± 0.15*
T. viridae	*8.00 ± 0.03*	*9.62 ± 0.18*
T. rubrum	*8.13 ± 0.21*	*9.71 ± 0.27*

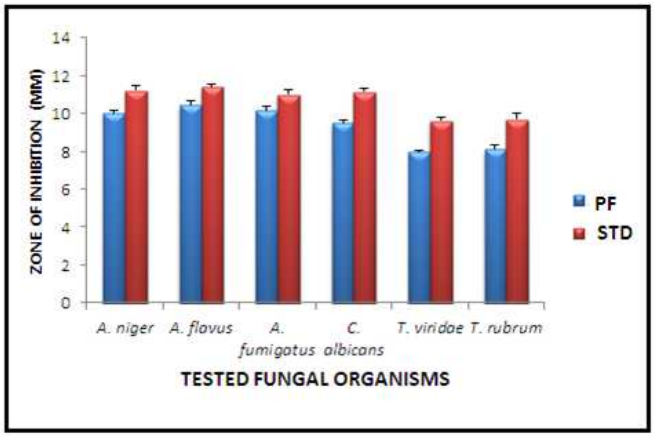

Fig 3: Antifungal activity of *A. stellatus*

Haemolytic activity:

The crude extract of *A. stellatus* showed pronounced haemolytic activity on chicken blood. The crude extract showed maximum haemolytic activity maximum at 1000µg/ml. Haemolytic activity was found to be increasing with increase in concentration as shown in the fig 3.

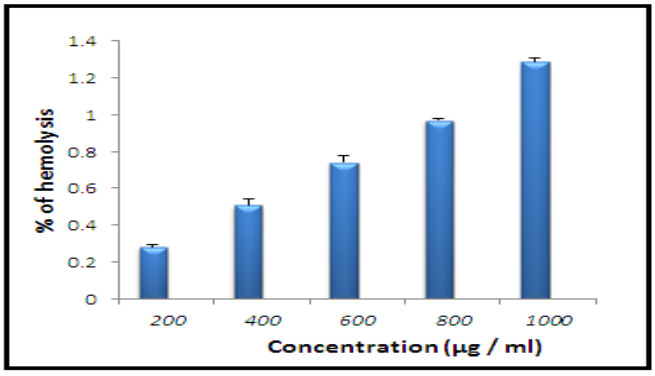

Fig 4: Haemolytic activity of *A. stellatus*

Cytotoxic activity against HeLa cell line:

Hela cell line was used to screen the cytotoxic activity of crude extract of *A. stellatus*. The crude extracts inhibited the growth of HeLa cell line in a dose dependent manner. It suppresses the growth of cells at the various concentrations after the treatment as shown in the table 3 and fig 5. The IC_{50} value (Concentration causing 50% inhibition of the growth of the cell was used as parameter for cytotoxicity. This test showed that the crude extract was toxic to the HeLa cell line and IC_{50} for Sample was found to be 64.86 µg /ml.

Table 3: Cytotoxic effect of *A. stellatus* on HeLa cell line

Concentration (µg/ml)	% of inhibition
1.5	1.42
3	5.12
6	11.21
12.5	19.26
25	24.81
50	41.32
100	63.21

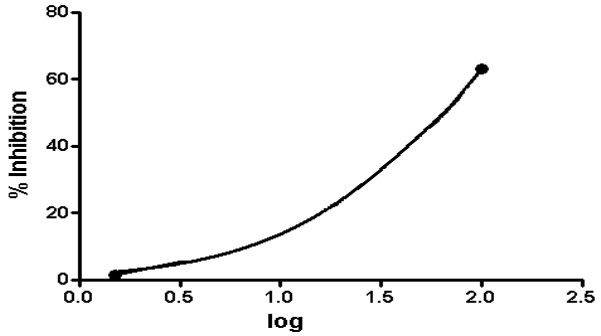

Fig 5: Cytotoxic effect of *A. stellatus* on HeLa cell line

Discussions

Biologically active compounds of varying degree of action are isolated from marine sources. Even then the marine ecosystem consists of largely unexplored domain for isolation of potential compounds. Widespread research is done to uncover the bioactive potentials of these compounds and some of them with potential antibacterial and antifungal activities are intensely being used as antibiotics against many diseases. Research is also being conducted on the characterization and biochemical properties of marine compounds. Large numbers of marine organisms produce toxins or poisons for defence mechanism or as secondary metabolites, which have shown effectiveness in treating many diseases. The biological interface between fish and their surrounding environment consists of a mucus layer comprising of biochemically diverse secretions from epidermal and epithermal cells (15, 16). The epidermal layer provides a mechanical protective function to be involved in osmoregulation and locomotion to play a possible immunological role (17, 18).

The antibacterial activity of fish has been known for many years and has been demonstrated in the mucous of several fishes. It was reported that epithelial tissues produce antimicrobial molecules which serve as the first line of a host defence against microbial invasion in vertebrates (19). In this study, antimicrobial activity of skin extract of *A. stellatus* has shown activity against various bacterial and fungal organisms. The antibacterial activity was found maximum *against E. coli and S. aureus* and minimum against *K. pneumoniae*, antifungal activity was observed maximum against *A. niger* and *A. flavus* and minimum activity against *T. viridae* by the skin extract of pufferfish *A. stellatus* . Similar result were also reported from the puffer fish *A. hispidus* from the Mandapam coast (20) from *A. immaculatus* from the Parangipettai coast (21) Which reports antibacterial activity against various pathogens. Tetrodotoxin producing bacteria in *A. hispidus* has showed haemolytic activity on chicken blood reported by Bragadeeswaran et al., 2010. (22). In this study, the chicken blood showed maximum haemolytic activity at 1000 µg/ml by the skin extract of *A. stellatus*. The viability of HeLa cell line was adversely affected upon adding the crude skin tissue extract of *A. stellatus*. The current study demonstrates, the skin extract of *A. stellatus* has shown the cytotoxic activity against HeLa cell line. Bragadeeswaran et al., (2010) also reported cytotoxic activity of crude bacterial extracts isolated from the skin of *A. hispidus*. Even the masked puffer fish, *A. diadematus* has shown antitumor activity against the Ehrlich ascites carcinoma cells (23). The results clearly indicate the bio toxin present in the fish is having potential that may be used for pharmaceutical needs. Though this toxin is a health threat in its original form due to its binding ability, yet it shows potentials as a drug in lower doses.

Conclusion

The present study clearly demonstrates the bioactive potential of the *A. stellatus.* Thus, it can be concluded that biotoxins from the *A. stellatus* has an excellent source for further development as a potential drugs.

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References

1) Garson MJ. Biosynthetic studies on marine natural products. Nat Prod Rep 1989; 6: 143-170.
2) Chew YL, Lim YY, Omar M, Khoo KS. Antioxidant activity of three edible seaweeds from two areas in South East Asia. Food Sci Technol 2007; 41: 1067–1072.
3) Rajamanikandan S, Sindhu T, Durgapriya D, Anitha JR, Akila S, Gopalakrishnan VK. Molecular docking and QSAR studies on bioactive compounds isolated from marine organisms into the MUC1 Oncoprotein. Int J Pharm Pharm Sci 2011; 3: 168-172.
4) Khora SS. Marine Fish-Derived Bioactive Peptides and Proteins For Human Therapeutics, Int J Pharm Pharm Sci 2013; 5(3): 31-37.
5) Ellis AE. Non-specific defense mechanisms in fishand their role in disease processes. Dev Biol Stand 1974; 49: 337-352.
6) Ravichandran S, Kumaravel K, Rameshkuma G, Ajithkumar TT. Antimicrobial peptides from the marine fishes. Res J Immunol 2010; 3(2): 146-156.
7) Seltzer Z, Dubner R, Shir Y. A novel behavioural model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 1990; 43(2): 205-218.
8) Cohen H. Puffer fish toxin for cancer pain, Atlantic breast cancer net (ABCN) newsletter, September 15th, 2004.
9) Alonso D, Khalilb Z, Satkunanthanb N, Livettc BG. Drugs from the Sea: Conotoxins as Drug Leads for Neuropathic Pain and Other Neurological Conditions. Mini Rev Med Chem 2003; 3: 785-787.
10) Song H, Li J, Lu CL, Kang L, Xie L, Zhang YY, Zhou XB, Zhong S. Tetrodotoxin alleviates acute heroin withdrawal syndrome: a multicentre, randomized, double-blind, placebo-controlled study. Clin Exp Pharmacol Physiol 2011; 38(8):510-4.
11) Khora SS. Toxicity studies on puffer fish from tropical waters. D. Ag. Thesis. Tohoku University, Sendai Japan, 1991.
12) Bauer AW, Kirby WMM, Sherris JC, Antibiotic susceptibility testing by a standardized single disc method, Am J Clin Pathol 1966; 45 (4): 493-496.
13) Bulmus V, Woodward M, Lin L, Murthy N, Stayton P, Hoffman A. A new pH responsive and glutathione-reactive, endosomalmembrane-disruptive polymeric carrier for intracellular delivery of biomolecular drugs. J Control Release 2003; 93:105-120.
14) Denizot RR and Lang. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol methods 1986: 5-22.
15) Pickering AS. The distribution of mucus cells in the epidermis of the brown trout *Salmo trutta* (L) and the char *Salvelinus alpinus* (L). J Fish Biol 1974; 6: 111-118.
16) Ellis A. Immunity to bacteria in fish. Fish Shellfish Immunol 1999; 9: 291-308.
17) Cameron A, Endean R. Epidermal secretions and evolution of venom glands in fishes. Toxicon 1973; 11: 401-410.
18) Fletcher T. Defense mechanisms in fish. In: D. Malins and J. Sargent (eds). Biochemical and Biophysical Perspectives in Marine Biology. London. Academic Press, 1978; pp. 189-222.

19) Ganz T. Defensins and host defense. Science 1999; 266: 420-421.

20) Mohana Priya K and Khora SS. Antimicrobial, Hemolytic and Cytotoxic activities of the Puffer Fish *Arothron hispidus* from the Southeast Coast of India. Int J Drug Dev Research 2013, 5(2): 317-322.

21) Kumaravel K, Ravichandran S, Sharmila Joseph, Manikodi D, Mauro Doimi, In vitro Antimicrobial Activity of Tissue Extracts of Puffer fish *Arothron immaculatus* Against Clinical Pathogens. Chinese J Nat Medicines 2011; 9 (6): 446–449.

22) Bragadeeswaran S, Therasa D; Prabhu K, Kathiresan K. Biomedical and pharmacological potential of tetrodotoxin-producing bacteria isolated from marine pufferfish *Arothron hispidus* (Muller, 1841). The J Venomous Animals Toxins Trop Diseases 2010; 16(3): 421-431.

23) Fouda FM, Anti-tumor activity of tetrodotoxin extracted from the Masked Puffer fish *Arothron diadematus*. Egypt J Biol 2005, 7, 1-13.

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