International Journal of Drug Development & Research
| April-June 2013 | Vol. 5 | Issue 2 | ISSN 0975-9344 |
Available online http://www.ijddr.in
Covered in Official Product of Elsevier, The Netherlands
SJR Impact Value 0.13 & H index 2

Full Length Research Manuscript

Antimicrobial, Hemolytic and Cytotoxic activities of the Puffer Fish *Arothron hispidus* from the Southeast Coast of India

K. Mohana Priya and Samanta S. Khora*
Medical Biotechnology Division, School of Biosciences and Technology, VIT University, Vellore - 632014, Tamilnadu, India.

Abstract

To evaluate the antimicrobial, hemolytic and cytotoxicity of toxin present in the puffer fish *Arothron hispidus* from the southeast coast of India. Liver, Skin, Muscle tissue extract of *A. hispidus* was extracted by using the acetic acid. In vitro antimicrobial test was done by disc diffusion method against seven bacterial and five fungal strains. Hemolytic and cytotoxicity test against HeLa cell line done by following the standard method. All the extract has shown inhibitory activity for antimicrobial test against bacterial and fungal strains, the skin extract has shown maximum zone against the *E. coli and A.niger* and the liver extract has shown minimum against *P. vulgaris* and *T. viridae*. Maximum hemolytic activity was observed in the skin extract of *A. hispidus*. In cytotoxic test, the maximum level of inhibition was observed on the growth of Hela 2 cell line by the skin extract of fish. The IC_{50} value of skin extract was found to be 1.78 mg/ml. These results indicated that the skin extracts of *A. hispidus* may have highly biologically active compound that can be further explored and utilized for welfare of mankind.

*Corresponding author, Mailing address:
Dr. Samanta S. Khora
Medical Biotechnology Division, School of Biosciences and Technology, VIT University, Vellore - 632014, Tamilnadu, India.
E. mail id: sskhora@vit.ac.in

Key words:
A. hispidus, Antimicrobial activity, Cytotoxicity, Hemolytic activity.

How to Cite this Paper:
K. Mohana Priya and Samanta S. Khora* "Antimicrobial, Hemolytic and Cytotoxic activities of the Puffer Fish *Arothron hispidus* from the Southeast Coast of India" Int. J. Drug Dev. & Res., April-June 2013, 5(2): 317-322.

Article History:----------
Date of Submission: 22-04-2013
Date of Acceptance: 06-05-2013
Conflict of Interest: NIL
Source of Support: NONE

INTRODUCTION

Terrestrial environment have relatively exhausted its resource for the pharmaceutical purpose. Recent decades, Marine ecosystem has been drawn attention of many scientists due to its biological and chemical diversity [1]. Marine environment acts as a huge

reserve to exploit natural products for the treatment of human and fish disease [2]. In last century, there has been a huge progress in the field of marine natural products and its chemistry [3, 4], So far approximately 7000 marine natural products have been reported from the marine organisms mainly invertebrates [5]. Among them, Fishes are the largest class of invertebrates. Due to the aquatic environment, fishes have some unique characteristics [3, 4]. Fishes are evolving with innate immune response to protect themselves against the infection [6].

Puffer fish belong to the family Tetraodontidae, order Tetraodontiformes commonly found in tropical seas is accumulating tetrodotoxin (TTX) in the body by the bioaccumulation via food chain. Tetrodotoxin, a sodium channel blocking compound mainly causes respiratory paralysis and intoxications [7, 8]. Traditionally, Japanese and Chinese are used to eat as delicacy and some part of the puffer fish as health tonic. Japanese researchers before World War 2 have used the crude puffer fish extract for treating migraines and menstrual cramps [9]. It has been shown a reducing symptom of withdrawal of heroin without any side effects. During last decades, TTX has been used as a tool for analyzing and characterizing the voltage gated sodium channel [10]. Recently, China and Canada have performed clinical trials for using TTX as an analgesic for reducing the pain in the cancer patients [11].

In India, studies on the puffer fish are very limited and it remains unexploitated. The aim of the present study is to evaluate the bioactive compound present in the puffer fish *A. hispidus* which is very common from the southeast coast of India.

MATERIALS AND METHODS

Specimen collection

Puffer fish specimens of *A. hispidus* (Muller, 1841) Family- Tetraodontidae, Order- Tetraodontiformes was collected from the fish landing station at Mandapam region near Ramasewarm at the latitude of 9 ·17 ◌̊ N – 70.9 E ◌̊ longitudes from the southeast coast of India in the month of September 2012. Then they were washed with sea water and transported to the laboratory in dry ice and was stored in the deep freezer at -20° C [12].

Extraction of samples

Specimen *A. hispidus* was thawed and dissected into tissues like Muscle, Liver, and Skin. Ten grams of each tissue was homogenized with 50ml of 0.1% acetic acid and were boiled in a hot water bath around 45° C for 10mins and cooled and centrifuged off. Then it was condensed using rotary evaporator at 60° C. Then it was stored at the deep freezer for further use at -20° C [13].

Tested strains:

Gram positive strains *Bacillus cereus, Staphylococcus aureus* and Gram negative bacteria *Escherichia coli, Klebsiella pneumoniae Pseudomonas aeruginosa, Proteus vulgaris, Vibrio chlorae* were used. Fungal strains *Candida albicans, Aspergillus niger, Aspergillus fumigatus, Trichophyton rubrum and Trichophython viridae* were used.

Antibacterial activity test

Antibacterial test of tissue extracts was performed *in vitro* using the agar disc diffusion

method according to Kirby with little modifications [14]. Sterile disks of 5mm in diameter were impregnated with 20µl of tissue extracts kept on the Muller Hinton agar plates previously swabbed with bacterial culture and incubated at 37° C for 24hrs. The diameter of the inhibition zones of the plates around the discs were measured and expressed in mm. All tests were performed in triplicate. Ampicillin was used as the positive control.

Antifungal activity test

Antifungal activity of tissue extracts was screened using the agar disc diffusion method

according to Mederios *et al.,* 2011 with little modifications [15]. Sterile discs of 5mm in diameter were impregnated with 20µl of tissue extracts kept on the Muller Hinton Agar plates previously swabbed

with fungal culture and incubated at 37° C for 72 hrs the diameter of the inhibition zones of fungal growth around the discs were measured and expressed in mm. Flucanozole was used as the positive control.

Hemolytic activity

Hemolytic assay was performed by the microtitre plate method of Prasad with little modifications [16]. Heparin used as an anticoagulant. A chicken blood was collected from the local slaughter house at Vellore and was centrifuged at 5000 rpm for 5mts, the supernatant was discarded and the pellet was suspended in normal saline (pH 7.4). The process was repeated thrice to obtain the erythrocyte suspension. The hemolytic test was performed in 96 well microtitre plates. Two fold dilution of the crude extract were made with 100 µl of saline. Then 100 µl erythrocyte suspensions were added to the wells. 100 µl of distilled water maintained as positive control. Then, the plate was gently shaken and it was allowed to stand for 3hrs at the room temperature and the results were observed. The uniform red color suspension in the well considered as positive hemolysis and a button formation in the bottom of the wells was considered as lack of hemolysis.

Cytotoxicity test

Hela cells were purchased from the NCCS (National Centre for Cell Sciences) Pune, India. The cells were sub cultured in a fresh flask containing Dulbecco Minimum Essential Medium (DMEM) with 10% fetal Bovine serum. The cells were rinsed in serum free medium and trypsin (0.1%) was added to remove the cell from substrate. The cells were counted and seeded on to a 96well microtitre plate at the rate of 200µg/ml and incubated overnight at 37° C in CO_2 incubator. 10mg/ml stock of the test samples was prepared in DMEM. The stock samples were serially diluted in DMEM 10% FBS. 100µl of the diluted cells were added to cells in triplicate wells containing 100µl of the medium. The well with 100µl of these DMEM was served as a negative control and 5µg/ml Doxorubicin was used as a positive control. Wells without any cells were used as a blank. The plates were incubated at 37° C in CO_2 incubators for 48hrs.

After 48hrs of incubation the plates were taken for the MTT cell viability test. 20µl of 5mg/ml MTT in PBS was added to each well and incubated at 37° C for 4hrs. Then medium was aspirated and replaced with 200µl of Dimethyl sulfoxide to each well. Then the plates were incubated for 10mts and then read at microplate reader at 570nm [17].

RESULTS

Antibacterial assay

The crude tissue extracts (Liver, Muscle, skin) of *A. hispidus* were screened against seven human pathogenic bacteria for testing their antibacterial activities. The inhibition zones of the extracts were compared with std Ampicilin for bacterial culture. The maximum zone was observed against the *E. coli* in the skin extract of *A.hispidus* and the minimum zone was observed against *P. vulgaris* in the liver extract as shown in Table 1 & Figure 1.

Table 1: Screening of antibacterial activity:

Organisms	STD- Ampicilin	Skin	Liver	Muscle
E. coli	12.8 ± 0.2	11.9 ± 0.9	11.1 ± 1.6	10.4 ± 0.1
K. pneumoniae	10.6 ± 0.3	10.1 ± 1.6	9.50 ± 1.7	9.10 ± 0.6
S. aureus	11.8 ± 0.3	11.1 ± 0.8	10.7 ± 0.8	10.0 ± 0.5
B. cereus	11.1 ± 0.4	10.7 ± 0.9	10.1 ± 0.8	9.60 ± 0.4
P. aeruginosa	10.2 ± 0.4	9.80 ± 0.3	9.20 ± 0.6	8.80 ± 0.9
P.vulgaris	11.7 ± 0.9	10.1 ± 0.6	9.60 ± 1.0	10.0 ± 0.1
V. chloreae	10.2 ± 0.4	9.80 ± 0.6	9.20 ± 1.0	8.70 ± 0.5

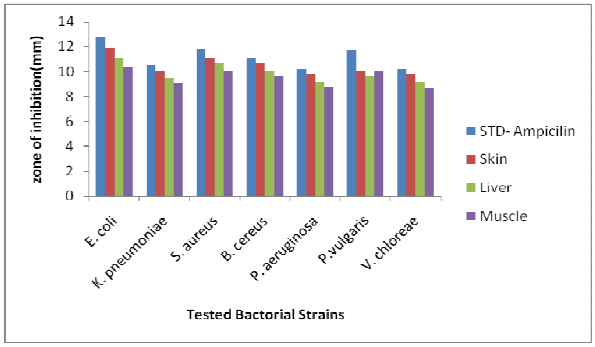

Figure 1: Antibacterial activity of Puffer fish *A. hispidus*

Antifungal activity:

The crude tissue extracts like liver, muscle, and skin of *A. hispidus* were screened against five human pathogenic fungi for testing their antifungal activities. The inhibition zones of the extracts were compared with standard Flucanozole for fungal culture. The maximum zone was observed against the *A. niger* in the skin extract of *A. hispidus* and the minimum zone was observed against *T. virdae* in the liver extract as shown in table 2 & Figure 2.

Table 2: Screening of Antifungal activity:

Tested Strains	STD- Flucanozole	Skin	Liver	Muscle
A. fumigatus	12.1 ± 0.3	11.7 ± 0.4	11.5 ± 0.6	11.3 ±0.8
A. niger	12.1 ± 0.3	12.0 ± 0.1	11.9 ± 0.2	11.7 ± 0.4
A. flavus	11.8 ± 0.4	11.3 ± 0.8	11.0 ± 0.8	11.6 ± 0.2
C. albicans	11.8 ± 0.4	11.6 ± 0.8	11.4 ± 0.8	11.1 ± 0.7
T. viridae	12.3 ± 0.1	11.8 ± 0.7	11.6 ± 0.7	11.0 ± 1.3
T. rubrum	12.2 ± 0.2	11.8 ± 0.7	11.5 ± 0.7	11.1 ± 1.1

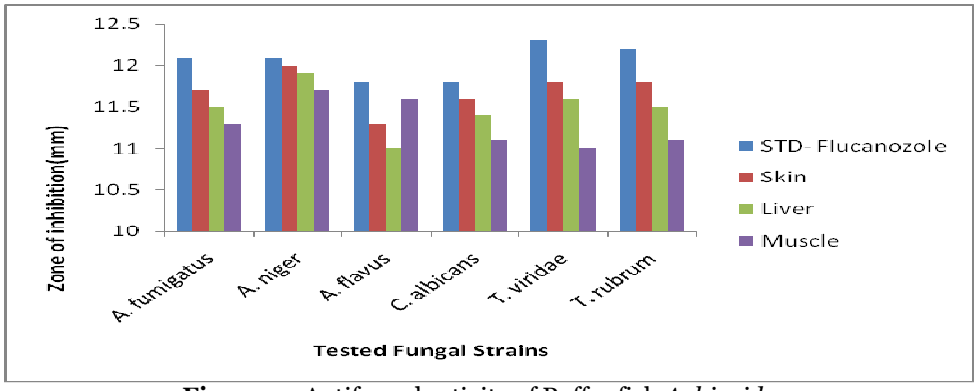

Figure 2: Antifungal activity of Puffer fish *A. hispidus*.

Hemolytic activity:

The crude tissues extracts of *A. hispidus* showed pronounced hemolytic activity on chicken blood. The highest hemolytic activity was shown by crude skin extract, and the liver and muscle extract showed moderate hemolytic activity.

Cytotoxicity test:

The viability of HeLa cells were observed upon adding the crude tissue extracts. The maximum activity against the HeLa cell line in the skin tissue extract of *A. hispidus*. When compared to other tissue extracts the IC_{50} values of the skin extract against the HeLa cell line was found to be 1.78mg/ml.

DISCUSSION:

Recent decades, Marine biotoxins has received much attention by various investigators all over the globe for their pharmaceutical potential. Fishes are living in challenging aquatic environment which contains a wide range of adverse effective compounds. The mucus secretions in the epidermis acts as a biological barrier for the fish protect from the environment [18]. Previous reports revealed that the components present in the epidermal secretion of the mucus acts as a barrier against pathogens and therefore may have a potential source of antimicrobial compounds [19]. Many researchers has been reported an endogenous peptides with antimicrobial activity from fish mainly from the skin and its secretions[20]. The antimicrobial property of mucus against the various pathogens has been demonstrated previously in rock fish [21], rainbow trout [22], and various species of cat fishes[23]. In this study, a pronounced antimicrobial activity has shown against various bacterial and fungal strains. The tissue extracts of *A. hispidus* shown activity against the bacterial and fungal strains. The antibacterial activity was observed maximum against *E. coli* by skin extract and minimum was found against *P. vulgaris* by liver extract. The antifungal activity

maximum was observed against *A. niger* and the minimumv against *T. viridae.* It goes along with the report of Kumaravel *et al.*, 2011 puffer fish. *Arothron immaculatus* from Parangipettai coast showed antibacterial activity against pathogens and that extracts not shown any activity against fungal pathogens [24]. In this study, the extracts have been shown activity against fungal strains. These variations occurred may be due to the physical parameters of the environment and the chemical composition in the fish. Chicken blood showed high hemolytic activity on the skin tissue extracts when compared to liver, muscle extract as same as reported by Bragadeeswaran *et al.,* 2010 has studied the hemolytic potential of tetrodotoxin producing bacteria in *A. hispidus* [25]. The pronounced cytotoxicity of skin extracts showed inhibitory activity against Hela cells. Antitumor activity of the masked puffer fish *Arothron diadematus* was studied by the Fouda *et al.,* 2005 [26]. The results clearly indicate the toxins present in the fish are having bioactive compounds that may be used for therapeutic needs.

CONCLUSION

In the present study the various parts of puffer fish has been examined for their bioactivity and in particularly the crude skin extract showed a broad spectrum of activities. These results revealed that *A. hispidus* skin has some valuable bioactive compound. However, it would have to be further subjected to analyze the extract compound present in it.

REFERENCES:

1) Chew YL, Lim YY, Omar M, Khoo KS, Antioxidant activity of three edible seaweeds from two areas in South East Asia. Food Science and Technology, 2007; 41 : 1067–1072.
2) Chellaram C, Gnanambal KME, Edward JKP. Antibacterial activity of the winged oyster *Pteria chinensis* (Pterioida: Pteridae). Indian J Mar Sci 2004; 33(4): 369-372.
3) Ellis A. Innate host defence mechanisms of fish against viruses and bacteria Dev Comp Immunol 2001; 25 (8): 827-839.
4) Plouffe DA, Hanington PC, Walsh JG, et al. Comparison of select innate immune mechanisms of fish and mammals Xenotransplantation 2005; 12 (4): 266-277.
5) Venkataraman K, Wafer M. Coastal and marine biodiversity of India. Indian Journal of Marine science 2005; 34(1): 57-71.
6) Noguchi T, Arakawa O. Tetrodotoxin: distribution and accumulation in aquatic organisms, and cases of human intoxication. Mar Drugs 2008; 6(2): 220-242.
7) Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 1990; 43(2): 205-218.
8) Lau FL, Wong CK,Yip SH. Puffer fish poisoning. J Accid Emerg Med. 1995; 12(1): 214-215.
9) Howard Cohen. Puffer fish toxin for cancer pain, Atlantic breast cancer net (ABCN) newsletter, September15th, 2004.
10) Tosteson MT, Effects of toxins on the voltage-gated Na+ channel, Proc. Third Int. Conf. Ciguatera, Portorico, Tosteson 1992; 2: 47-54.
11) Alonso D, Khalilb Z, Satkunanthanb N & Livettc BG. Drugs from the Sea: Conotoxins as Drug Leads for Neuropathic Pain and Other Neurological Conditions. Mini Reviews in Medicinal Chemistry 2003; 3: 785-787.
12) Hashimoto Y, Marine toxins and other bioactive metabolites, Japan Scientific society press, Tokyo 1979.
13) Kawabata T, Food hygiene examination manual Assay method for Tetrodotoxins. Jap. Food Hyg. Assoc. Tokyo, Japan, 1979; 1: 223-24.
14) Bauer AW, Kirby WMM, Sherris JC, et al. Antibiotic susceptibility testing by a standardized single disc method , Am J Clin Pathol 1966; 45 (4): 493-496.
15) Medeiros RTS, Evaluation of antifungal activity of *Pittosporum undulatum* essential oil against *Aspergillus flavus* and aflatoxin production. Ciência e Agrotecnologia 2011; 35(1):71-76.
16) Prasad KP, Venkateshvaran K. Microhemolytic assay. In: Venkateshvaran K, Prasad KP, editors.

Training manual on advance techniques in marine biotoxinology. Mumbai: CAS in Fisheries Science, Central Institute of Fisheries; 1997; 41-2.

17) Amir Zamiri-Akhlaghi1, Hasan Rakhshandeh1, Zahra Tayarani-Najaran1, Seyed Hadi Mousavi Study of cytotoxic properties of *Rosa damascena* extract in human cervix carcinoma cell line. Avicenna Journal of Phytomedicine 2011; 1(2): 74-77.

18) Shephard K L, Functions for Fish Mucus, Reviews in Fish Biology and Fisheries, 1994; 4: 401.

19) Park IY, Park CR, Kim MS, Kim SC, Parasin I, An antimicrobial peptide derived from Histone H2A in the cat fish *Parasilurus asotus*, FEBS Lett, 1998; 437:258-262.

20) Knouft JH, Page L, Plewa MJ. Antimicrobial egg cleaning by the fringed darter (Paciforms: Peridae: *Etheostoma crossosterum*): Implication of a novel component of parental care in fishes. Proc R Soc London Ser 2003; B 270: 2405-2411.

21) Kitani Y, Kikuchi N, Zhang GH, Ishizaki S, Shimakura K, Shiomi K, Nagashima Y.Antibacterial action of Lamino acid oxidase from the skin mucus of rockfish *Sebastes schlegelii*. Comp Biochem Physiol 2008; 149: 394-400.

22) Raida MK, Buchmann Innate immune response in rainbow trout (*Oncorhynchus mykiss)* against primary and secondary infections with *Yersinia ruckeri*. Dev Comp Immunol 2009; 33: 35-45.

23) Ramasamy Anbuchezhian, Gobinath, Ravichandran, Antimicrobial Peptide from the Epidermal Mucus of Some Estuarine Cat Fishes. World Applied Sciences Journal 2011; 12 (3): 256-260.

24) Bragadeeswaran S; Therasa D; Prabhu K; Kathiresan K. Biomedical and pharmacological potential of tetrodotoxin-producing bacteria isolated from marine pufferfish *Arothron hispidus* (Muller, 1841). J. Venom. Anim. Toxins incl. Trop. Dis 2010; 16(3): 421-431.

25) 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 Journal of Natural Medicines 2011; 9 (6): 446–449.

26) Fatma M Fouda, Anti-tumor activity of tetrodotoxin extracted from the Masked Puffer fish *Arothron diadematus*. Egyptian Journal of Biology 2005; 7: 1-13