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RESEARCH ARTICLE

PRELIMINARY SCREENING OF SECONDARY METABOLITES AND BRINE SHRIMP LETHALITY BIOASSAY OF WARM-WATER EXTRACT OF PUFFER FISH ORGANS TISSUES, *TETRAODON CUTCUTIA,* AVAILABLE IN BANGLADESH.

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ABSTRACT

This study is aimed to establish the invitro screening of secondary metabolites namely alkaloids, steroids, alcoholic compounds, glycosides, and amide group containing compounds from warm-water extracts of puffer fish dorsal flesh, head, tail and liver tissues by conventional laboratory procedures. In secondary metabolites screening, the warm-water extract of puffer fish liver tissues excepting fish dorsal, head, and tail extract showed positive test of alkaloids, gycosides and alcoholic group test while gave negative test of steroid, amide group containing compounds. This property is found to similar to TTX, which was isolated from puffer fish available in Japan. On the other hand, fish extracts from dorsal, head, and tail showed the positive test against glycosides, alcohol, and amide but not for steroids, and alkaloids. To observe bioactivity, the warm-water crude extracts were prepared at the concentration of 02, 04, 08, 16, 32, and 64 µg/ml, and their bioactivity were tested using Brine shrimp lethality bioassay against larva Artemia Leach. The LC_{50} (µg/ml) of the different extracts were calculated by prohibit analysis. The fish tissues extract exhibited toxicity against larva Artemia Leach, and LC_{50} were 0.75, 0.90, 1.30, and 1.45 µg/ml but other extracts of puffer fish did not show toxicity. Further characterization is still needed for the identification of compound found in puffer fish liver extract, and would be considered as the potential candidates for new drug research.

KEY WORDS: Secondary metabolites, *Tetraodon cutcutia*, and bioactivity

INTRODUCTION:

Bangladesh is a land of river and rich in marine resources. It is generally accepted that ten thousands species of marine fishes and shellfish inhabit in the sea. Many of them are used as an important food source. However, at least several hundred species are regarded as toxic and many causes human poisoning, when ingested behind safety level. Some examples of marine toxins are paralytic shellfish toxin, diuretic shellfish poison, coguatic toxin as well as tetrodotoxin.

The puffer fish takes its name from its tendency to puff its throat with water or air when predator's approach or a threatening situation arises, giving the fish a balloon-like appearance. In different parts of the world the puffer fish has different name such as blow fish, swell fish, balloon fish, toad fish, and globe fish. The eyes and internal organs of many Tetraodontidae are highly toxic, but nevertheless the meat of some species is considered a delicacy in Japan and Korea [1]. The name Fugu is used both for the fish that are eaten and for their meat. The Korean term for this fish is "boh-guh" fish. Before preparing the fish, Japanese chefs are required to face an examination board and get a special license. Despite these precautions about 100-200 people get fugu fish poisoning every year, and about half of the cases result in death [2-5]. Death due to puffer fish poisoning have also been reported in Singapore, Hong Kong, and Australia [6-8]. Puffer fishes are also popular with the Japanese community in the United States [9]. In April 1996, however, three cases of puffer fish poisoning in California were reported. At present personal importation of the fish into the United States is probit [10].

In Bangladesh, the puffer fish is popularly known as potca fish. Because of low cost and availability, puffer fish is frequently eaten, particularly by the people in poor rural communities. But in April 2002, 37 people from eight poor families were admitted to Khulna Medical College Hospital eight of then died [11]. Six people of a family were died on eating puffer fish at Patuakhali in 2001. In 16 November 1998, eight people died due to its toxin due to lack of

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knowledge. Recently (14 may, 2005, daily news papers) a fatal out break of puffer fish poisoning occurred in Vola district of our country where 6 peoples died [12]. Three of ten fatalities, occurred in 1950 to 1990 in USA and four in Hawaii in 1903 to 1925 [13-15], due to toxin. In, Japan from 1974 to 1983, there were 664 reported cases of puffer fish poisoning, with 179 fatalities [16-17]. In this study, we screen potential bioactive compounds from puffer fish extracts, and also report on it's cytoxicity against larvae Artemia Leach, and find out the anti-tumor relationship of identified bioactive compounds.

METHODS:

COLLECTION AND IDENTIFICATION OF THE PUFFER FISH:

The puffer fish was collected from Rupsha fish market-Khulna and Mongla port. It was mainly caught form the river sourrounding Shundarban in the month of November, 2006. Puffer fish was identified with the help of the department of Zoology (Fisheries Section), University of Rajshahi and used for experimental purpose. Systematic position of puffer fish is given below

Kingdom: Animalia
Phylum: Chordata
Sub-phylum: Vertebrata
Class: Osteichthyes
Order: Tetraodontiformes
Family: Tetraodonitidae
Genus: Tetraodon
Species: cutcutia
Full scientific name: *Tetraodon cutcutia*
Local name: Potca, fotca, Tepa, Cutcutia
Japanese name: Fugu / puffer fish

CHEMICALS: All chemicals used in the preparation of puffer fish organs extracts were analytical grade supplied by Marck, Germany.

FISH PULVERIZATION: First of all, the collected puffer fishes were cleaned well with tap water, and the dorsal, head, tail, and liver were separated and cut into small pieces and then macerated into paste using mortar and pestle. The homogenates were filtered with the help of muslin cloth.

EXTRACTION: The extracts of different parts of puffer fish were obtained maceration using warm-water followed by partition using n-hexane, chloroform, ethyl acetate , and then water extracts of fish dorsal, head, tail, and liver were collected by filtration using Whatman no-41 filter paper to complete extraction. Lastly, water extracts were freeze-dried to get solid crude materials for pursues our study.

SECONDARY METABOLITES SCREENING: Preliminary qualitative secondary metabolites screening were carried out with the following methods.

STEROIDS: 100 mg of the extracts were dissolved in 5 ml of chloroform and a few drops of concentrated sulphuric acid are added to it followed by the addition of 2-3 drops of acetic anhydride. Development of a light green color indicates the presence of the steroid [18].

ALKALOIDS: About 100 mg of the extracts were taken on a watch glass, then 2 ml of HCl is added and the mixture is stirred with a glass rod. Half drops of Dragendorff's or Mayer's reagent is added to the solution and formation of a brick red precipitates on the watch glass indicates the presence of alkaloid [19].

ALCOHOLS: 100 mg of the extracts were dissolved in 0.5 ml of dioxane. The solution is mixed with 0.5 ml of ceric ammonium nitrate reagent, and shaken well. Development of a yellow to red color indicates the presence of alcoholic hydroxyl group [20].

CARDIAC GLYCOSIDES: About 100 mg fish extract was dissolved in 1ml glacial acetic acid containing one drop of ferric chloride solution, and this was then underlayer with 1 ml of conc. H_2SO_4. A brown ring obtained at the interface indicated the presence of a deoxy sugar characteristic of cardenolides [21].

AMIDE TEST: 100 mg of the extracts were dissolved in 3 ml of 20% NaOH and the solution is boiled for 15-20 minutes. Then the liberation of ammonia gas indicates the presence of amide group. The ammonia gas is identified by its pungent odor and also the gas turns red litmus to blue [22].

BRINE SHRIMP LETHALITY BIOASSAY: The comparative toxicity of different parts of puffer fish was examined by Brine shrimp lethality Bioassay [23]. The samples were prepared by dissolving 0.1mg of each extract in 5 ml sterile distilled water, and appropriate amount of 5% DMSO solution was transferred into the six vials to get final concentration of 02, 04, 08, 16, 32, and 64 µg/ml respectively making the volume up to 5 ml by seawater (artificial). For the negative control test, we were used 1ml 5% DMSO, and 4 ml seawater in another vial.

HATCHING THE BRINE SHRIMP: Brine shrimp eggs **(***Artemia salini)* were hatched in artificial seawater prepared by adding 38g of commercial sea salts (NaCl) in 1 liter sterile distilled water, and specific gravity was 1.018 as measured with a hydrometer. To be sure seawater, starting pH was adjusted to 8.0-9.0 by adding Epson salt. The following parameters were also setup for appropriate hatching of brine shrimp eggs.

Temperation: Optimum temperature for a 24 hrs complete hatch was 80-82^{0}F or 26-28^{0}C.

Light: Illuminating is necessary to trigger the hatching mechanism within embryo during the first few hrs of incubation. Maintaining a light source during the entire incubation period was recommended.

Aeration: A minimum of 3 ppm dissolved oxygen during the incubation was maintained because of constant aeration is necessary to keep eggs in suspension and to provide sufficient oxygen levels for the eggs to hatch.

The brine shrimp eggs (1 g/L) were sprinkled into cone or V bottomed container and supplemented with 5 mg dried yeast / L seawater, and after 48 hrs incubation, nauplii (Larvae) were collected by pipette from the lighted side whereas their shells were left in another side, and were ready for bioassay.

BIOASSAY: Fifteen (15) brine shrimp nauplii (Larvae) were collected, and transferred into seven experimental vials containing tested extracts at different concentration, and a drop of yeast suspension (3mg/ml seawater) was added to each vial. After 24 hrs experimental test tubes were observed and the number of survived nauplii in each test tube was counted, and results were recorded.

DATA ANALYSIS: The percentage of lethality of brine shrimp larvae was calculated at each concentration by probit analysis on a Finney computer program to determine the LC_{50}.

RESULTS:

The secondary metabolites screening and qualitative estimation of puffer fish dorsal, head, tail, and liver tissues extract studied showed that liver extract exhibited characteristics test of alkaloids, alcohols, cardiac glycosides, and negative tests for steroids. On the other hands, dorsal, head, and tail extracts were assigned with positive tests for alcohols, steroids, and negative for alkaloids test, as shown in table 1.

Table 1: Secondary metabolites of the different tissue extracts of puffer fish

Fish crude extract	Alkaloids	Steroids	Alcohol	Amide	Glycosides
Liver	+	-	+	-	+
Dorsal	-	-	+	+	+
Head	-	-	+	+	+
Tail	-	-	+	+	+

Note: "+" indicates presence and "–"absence

In brine shrimp lethality bioassay, the liver extract showed the highest cytotoxicity against *Artemia salina* larvae (nauplii) as compared to other crude extracts like dorsal, head, and tail resulting 50% lethal cytotoxicity dose (LC_{50}) of 0.97, 0.90,1.30,and 1.45 µg/ml were found respectively in liver, head, dorsal, and tail extracts of puffer fish, as shown in table 2.

Table 2: Brine shrimp lethality bioassay of water extracts of puffer fish liver, dorsal, tail, and head after 24 hrs treatment.

Fish extracts	Conc. of sample (µg/ml)	Log conc.	LC_{50} (µg/ml)
liver	02,04,08,16,32,64	0.301, 0.602, 0.903, 1.20, 1.50, 1.80	0.75
Head	02,04,08,16,32,64	0.301, 0.602, 0.903, 1.20, 1.50, 1.80	0.90
Dorsal	02,04,08,16,32,64	0.301, 0.602, 0.903, 1.20, 1.50, 1.80	1.30
Tail	02,04,08,16,32,64	0.301, 0.602, 0.903, 1.20, 1.50, 1.80	1.45

DISCUSSION:

In Japan, they have been isolated a alkaloids (TTX) from puffer fish showed characteristics reaction like alkaloids test of liver extract from *Tetraodon cutcutia*, and recognized as a potent neurotoxin produced in fish intestinal bacteria.This neurotoxic alkaloid also assigned in USA, Australia etc even if in Bangladesh there are some life threatening phenomina [24] but there is no authentic documents of why puffer fish consumer became died. According to preliminary secondary metabolites screening, it is cleared that the toxicity of puffer fish, *Tetraodon cutcutia*, against life depends on the presence of alkaloids. The brine shrimp lethality bioassay has been exploited extensively in the preliminary screening of the crude extracts and/or isolated compounds to examine the toxicity to Artemia salina larvae, which also provides a possible cytotoxicity of sample molecules. In brine shrimp lethality bioassay, we found that the cytotoxic activity of fish crude extracts to *Artemia salina* larvae at different degree, and the liver extract showed most cytotoxic activity as comparable to other extracts of puffer fish due to the presence of alkaloids but other fish extracts contain glycosides compound excepting alkaloids. So the dorsal, head, and tail extracts showed their cytotoxic activity due

to the presence of strong glycosides compounds, and the lethal doses of the different fish extracts were determined graphically, and showed that fish liver extract was 0.97 µg/ml as comparable less than the other crude fish extracts. The cytotoxicity study in vitro exhibited by the fish extracts tested is a primary hint of in vivo anti-cancer activity. Interestingly, the concentrations of cardiac glycosides used for cancer treatment are extremely close to those found in the plasma of cardiac patients treated with the same drugs, suggesting that the anticancer effects of these drugs are exerted at non-toxic concentrations. Cardiac glycosides constitutes of a large family of naturally derived compounds, the core structures of which contain a steroid nucleus with a five-membered lactone ring (cardenolides) or a six-membered lactone ring (bufadienolides) and sugar moieties, [25] namely digoxin, digitoxin, ouabain, and oleandrin. Of them digitoxin and digoxin, are well-known cardiac glycosides, and acts as an inhibitors of the plasma membrane Na^+/K^+-ATPase that are clinically used for the treatment of heart failure. The positive inotropic effects of cardiac glycosides reduces the active counter-transportation of Na^+ and K^+ across the cell membrane, resulting an increase in the intracellular Na^+ concentration, and a decrease in the intracellular K^+ concentration, and finally a successive increase in cardiac contraction [26]. From previous evidence suggests that breast cancer patients who were treated with digitalis have a significantly lower mortality rate, and their cancer cells had more benign characteristics than those from patients not treated with digitalis [27]. Additionally, studies have suggested that cardiac glycosides target cancer cells selectively [28]. These promising findings have advanced considerable attention in the field of anticancer research, and furthermore studies on the anticancer properties of these compounds have been accompanied. These analyses explored not only digoxin and digitoxin but also other related cardiac glycosides, such as ouabain, oleandrin, proscillaridin A, and bufalin [29].Various mechanisms of action, including the inhibition of cancer cell proliferation, the induction of apoptosis, and chemotherapy sensitization, have been reported in a large number of published articles that sustenance the promising use of these compounds for cancer treatment [30]. However, further clinical studies are still ongoing to better characterize the pharmacological and safety issues associated with these compounds from puffer fish tissues. So, the results obtained in the present study authenticated that isolated components from puffer fish avoiding its alkaloids contents having propitious anti-tumor activity which could serve for further cytotoxicity study in animal model, and find the possible relationship between brine shrimp lethality bioassay and bioactivity of puffer fish organs extract.

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CONFLICT OF INTEREST:

We have no any conflict of interest.

REFERENCES:

1. Tsunenari S, Uchimura Y, Kanda M. Puffer poisoning in Japan, a case report. J Forensic Sci 1980; 25: 240-245.
2. Ellis RM, Jelinek GA. Never eat an ugly fish: three cases of tetrodotoxin posoning from Western Australia. Emerg Med 1997; 9: 136-142.
3. Torda TA, Sinclair, E, Ulyatt DB. Puffer fish (tetrodotoxin) poisoning: clinical record and suggested management. Med J Aust 1973; 1:599-602.
4. Field J. Puffer fish poisoning. J Accid Emerg Med 1998; 15: 334-336.
5. Duncan C. A case of toadfish poisoning. Med J Aust 1951; 2:673-675.
6. Tibballs J. Severe tetrodotoxin fish poisoning. Anaesth Intensive Care 1988; 16:215-217.
7. Cleland JB. Injuries and diseases in Australia attributable to animals (except insects). Med J Aust 1924; 2:339-345.
8. Kanchanapongkul J. Puffer fish poisoning: clinical features and management experience in 25 cases. J Med Assoc Thai 2001: 84:385-389.
9. Yang CC, Liao SC, Deng JF. Tetrodotoxin poisoning in Taiwan: an Analysis of poison center data. Vet Hum Toxicol 1996:38:282-286.
10. Kan SK, Chan MK, David P. Nine fatal cases of Puffer fish poisoning in Sabah, Malaysia. Med J Malaysia 1987; 42:199-200.
11. Rahman, A K M; 2005. *Freshwater Fishes of Bangladesh*, 2nd Edition. Zoological Society of Bangladesh, Department of Zoology, University of Dhaka, Dhaka, Bangladesh, XVIII+394 pp.
12. Talwar, P.K. and A.G. Jhingran; 1991. *Inland Fishes of India and Adjacent Countries*. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, India. LIV + 1158 pp.
13. Ebesu, J.S.M., Noguchi, T., and Hokama, Y., Fish Poisoning Due to Tetrodotoxin, In “Food Born Disease” (ed. by Hui), Science and Technology system, 2000.

14. Reddy, C. S., and Hayes, A. W., Food-born toxicants. In: "Principles and Methods of Toxicology", 2nd ed. (a. W. Hayes, ed.) Raven, New York, 81 P., 1989.

15. Large, W.R., Puffer Fish poisoning, Am. Fam. Physician, 42: 1029, 1990.

16. Tsuda, K., Ikuma, S., Kawamura, M., Tachikawa, R., Sakai, K., Tamura, C., and Amakasu, O., Tetrodotoxin. VII. On the Structures of Tetrodotoxin and its Derivatives, Chem. Pharm. Bull. Jmp., 12: 1357-1374, 1964.

17. Finar, I.L., "Organic Chemistry", Vol-2, 5th ed. p-518, 767-788; 1983.

18. Feigl, F., "Spot tests in organic Analysis" 7th ed. Elsevier publishing Company, London, p-175; 1966.

19. Pal and Chakraborty, "B.Sc. Practical chemistry", 2nd ed. p-277-278, 1987.

20. Vogel, I.A., "A test book of organic chemistry including quantitative organic analysis" 3rd ed. P. 1058, long man, London 1967.

21. Alfed Burger, medicinal chemistry 2nd ed. weley-inter science, publisher, Inc. New York. P-4, 6, 12, 314-319, 623-615, 389, 1960.

22. Meyer, Ferrigni, Putnam, Jacobsen, Nichols, Mc. Laughlin, 1982, Brine shrimp: A convenient General Bioassay for Active Plant constituents , Plant Medica, Vol-45

23. Odediyi,A. and J.A Sofowora, 1978. Phytochemical screening of Nigerian medicinal plants Part II Lioydia.403:234-246.

24. Mijatovic T, Ingrassia L, Facchini V, Kiss R. Na+/K+-ATPase alpha subunits as new targets in anticancer therapy. Expert Opinion on Therapeutic Targets 2008;121403-1417.

25. Böhm M. Digoxin in patients with heart failure. The New England Journal of Medicine. 1997;337 129-130.

26. Stenkvist B. Is digitalis a therapy for breast carcinoma? Oncology Report 1999;6493-496.

27. Gupta RS, Chopra A, Stetsko DK. Cellular basis for the species differences in sensitivity to cardiac glycosides (digitalis). Journal of cellular physiology 1986;127 197-206.

28. López-Lázaro M. Digitoxin as an anticancer agent with selectivity for cancer cells: possible mechanisms involved. Expert Opinion on Therapeutic Targets 2007;111043-1053.

29. Huang YT, Chueh SC, Teng CM, Guh JH. Investigation of ouabain-induced anticancer effect in human androgen-independent prostate cancer PC-3 cells. Biochemical Pharmacology 2004;67 727-733.

30. Tamao Noguchi, Joanne S. M. Ebesu. Puffer poisoning: Epidemiology and treatment. Toxin Reviews. 2001; 20:1 - 10

