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Biomedical and pharmacological potential of tetrodotoxin-producing bacteria isolated from marine pufferfish *Arothron hispidus* (Muller, 1841)

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Abstract: Specimens of the pufferfish *Arothron hispidus* collected at Parangipettai, on the southeast coast of India, were subjected to bacterial isolation and identification. Three species were identified, namely *Bacillus* sp., *Kytococcus sedentarius* and *Cellulomonas fimi*. Partially-purified microbial filtrates exhibited hemolytic activity on chicken and human erythrocytes of O, B and AB blood groups, with maximum activity of 32 HU. The microbial filtrates also presented ATPase, Mg^{2+}-ATPase, $Na^{+}K^{+}$-ATPase and AchE enzymatic activities of positive neuromodulation in *Kytococcus sedentarius* with 1300, 300.1, 1549.98 and 140.55%, in *Cellulomonas fimi* with 620, 300, 10 and 128.42%, and in *Bacillus* species with 40, 200, 849.98 and 158.69%, respectively. Toxicity symptoms were observed when the bacterial filtrate was intraperitoneally injected into mice. The bacterial filtrate caused adverse effects on viability of the mouse muscle cell line (L929) and leukemia cell line (P388). Maximum level of inhibition was observed on the growth of L929 cell line. *Bacillus lentimorbus* inhibited the cell line from 84.03 to 94.43% whereas *Bacillus* species inhibited the growth in a range between 77.25 and 86.16% at the lowest dilution.

Key words: *Arothron hispidus*, tetrodotoxin, bioassay, ATPase and AchE enzymatic activities, anticancer activity.

INTRODUCTION

Tetrodotoxin (TTX) is the best known marine toxin due to its frequent involvement in fatal food poisoning, unique chemical structure and specific action of blocking sodium channels of excitable membranes (1, 2). The toxin derives its name from the pufferfish family (Tetraodontidae) and occurs widely in the terrestrial and marine animal kingdom (3-6). Tetrodotoxin is a powerful neurotoxin that can cause death in 60% of humans (7). A human has to ingest only a few milligrams to produce a fatal reaction to the toxin (8). In recent years, tetrodotoxin has been utilized as a pharmacological probe.

As a defense mechanism, pufferfish have the ability to inflate rapidly, filling their extremely elastic stomach with water (or air when outside the water) until they are almost spherical in shape. The bacteria associated with some pufferfish produce powerful neurotoxin in their internal organs making them an unpleasant, possibly lethal, meal for any predator (9).

MATERIALS AND METHODS

Specimen Collection

The puffer fish *Arothron hispidus* (Muller, 1841) (Vertebrata: Actinopterygii: Tetraodontiformes: Tetraodontidae) was collected from the Annankoil fish landings at Parangipettai (Lat. 11° 29' N; Long. 79° 46' E) southeast coast of

India, and immediately brought fresh to the laboratory (1 km). Skin, intestine and liver tissues were aseptically excised for isolation of microbes.

Isolation and Identification of Bacteria

ZoBell marine agar 2216 (Himedia, Mumbai) was used for bacterial isolation. The agar plates were prepared in sterilized dishes with 15 g of agar dissolved in 1.0 L of ZoBell 2216 medium, adjusted to a pH of 7.2. One gram of pufferfish skin, liver and intestine each was homogenized and suspended in 9 mL of sterile sea water. The samples were serially diluted for isolation of bacterial strains by spreading 0.1 mL of the diluted samples on agar plates and the result was maintained at 28°C for 5 to 14 days to permit bacterial growth. Eventually, mixtures of colonies were formed on each plate, after which they were purified by the streak plate method on another new ZoBell 2216 agar plate until discrete colonies were obtained. Each discrete colony was further sub-cultured several times to ensure that it was a pure culture before bacterial identification using the Microbial Identification System (MIS version n. 4.5, Microbial Identification Inc., USA), which identified the whole cell fatty acid profiles between the bacterial samples and standards.

Identification by the Microbial Identification System

The microbial identification system relies on qualitative and quantitative analysis of the culture strains identified by the fatty acid composition of the organism (MIS version 3.8). Moreover, a gas chromatograph (Agilent GC 6890N®, Agilent Technologies, USA) equipped with a flame ionization detector and a 2-meter HP Ultra 2 capillary column (HP 6890N®, Agilent Technologies, USA) (inner diameter, 0.2 mm; film thickness, 0.33 mm) coated with 5% phenyl methyl silaxane were used. The rate of hydrogen carrier gas flow was maintained at 30 mL/minute. Fatty acid methyl esterases (FAMEs) were identified by the Microbial Identification (MIDI) calibration standard software. The pure culture strains from the third quadrant streaking (harvesting) culture were transferred into a tube with a Teflon-lined screw cap. The microbe was identified based on the fatty acid profile.

Isolation and Partial Purification of TTX from Bacterial Cultures

The pure isolate was inoculated with a sterile loop inside a capped 1,000-mL flask containing 500 mL of sterile Zobell broth medium for culturing at 25°C in an incubator for ten days in darkness without aeration (4); subsequently, it was subjected to TTX detection. The isolation and purification of TTX from bacterial cultures were basically the same procedures as those used for pufferfish tissues, briefly described by Yu (10), in which each broth culture was centrifuged at 3,000 rpm for 15 minutes to remove the precipitated bacterial cells.

The supernatant was evaporated under reduced pressure to a volume of water-washed activated charcoal under agitation and filtered through a funnel. The charcoal on the funnel was thoroughly washed with distilled water and the TTX adsorbed was eluted three times with three volumes of 1% acetic acid in 20% aqueous ethanol. The eluent was evaporated and lyophilized. The lyophilized toxin extract was dissolved in 10 mL of 0.03 M acetic acid and subjected to gel filtration through a column (2 x 50 cm) of Bio-Gel® P-2 (Bio-Rad Laboratories, USA) equilibrated with 0.03 M acetic acid. Toxic fractions were combined and concentrated at reduced pressure to a final volume of 10 mL which was subjected to further toxicity bioassay.

Hemolysis Study

The microbial crude extracts were assayed on chicken and human (A, B, AB and O) erythrocytes as described by Prasad and Venkateshvaran (11). Samples of chicken blood were obtained from a nearby slaughterhouse in Parangipettai, while the human blood was obtained from Sheena Clinic, Andheri (W), Mumbai, using 2.7% ethylenediaminetetraacetic acid (EDTA) solution as an anticoagulant at 5% of the blood volume, and brought to the laboratory. The blood was centrifuged thrice at 5,000 rpm for five minutes. A 1% erythrocyte suspension was prepared for hemolysis study.

The hemolytic test was performed in 96-well 'v' bottom microtiter plates. Serial two-fold dilutions of the crude toxin/fractions were made in 100 µL of normal saline. Then, 100 µL of 1% erythrocyte was added to all the wells. The positive and negative controls were maintained by adding 100 µL of distilled water and 100 µL of normal saline,

respectively, to the 1% red blood cell suspension. The plate was gently shaken and allowed to stand for three hours at room temperature. Presentation of uniform red-color suspension in the wells was considered to constitute positive hemolysis and a button formation in the bottom of the wells constituted an absence of hemolysis. The reciprocal of the highest dilution of the crude toxin showing the hemolytic pattern (hemolytic unit) was divided by the protein content to obtain the specific hemolytic unit.

Mouse Bioassay for Lethality

All animal bioassays were carried out by following the statement of the Institutional Ethics Committee (regulation number 160/1999/CPCSEA/11.01.2008) of the Rajah Muthiah Medical College, Annamalai University. The bioassay for lethality was done by using clinically healthy male albino mice weighting 20 ± 2g that were maintained in a healthy condition in the laboratory. Mice in triplicate sets were challenged intraperitoneally with 0.25, 0.50, 0.75 and 1.0 mL of the crude toxin, dissolved at 5 mg/mL and 1.0 mL each of the 11 fractions. A control was maintained in each case by injecting an equal volume of PBS (pH 7.4). The times of injection and death, in addition to behavioral changes before death, were recorded.

Neuromodulatory Activity

In Vitro Evaluation of the Effect of the Toxins on Mouse Brain ATPase Enzyme

P_2 fraction preparation

P_2 fraction (mitochondrial nerve endings) from male mouse (20 ± 2 g) brain was prepared by following the method of Green *et al.* (12). Brain tissue collected from the male albino mouse (weighting 20 ± 2 g) was homogenized in ice cold sucrose solution (0.32 M) and centrifuged (Sorvall Super T-20® refrigerated centrifuge, USA) at 2,500 rpm for 15 minutes at 4°C to remove cell debris, nuclei and plasma membrane fragments. Again, the supernatant was centrifuged at 15,000 rpm for 20 minutes at 4°C. Then, the supernatant was discarded and the pellet was dissolved in sucrose solution and repeated an additional two times. It was washed once again in the same fashion and the resultant pellet was dissolved in the sucrose solution depending upon the pellet size and kept in deep freezer as enzyme source.

ATPase assay

The ATPase assay for inorganic phosphate was performed according to Lowry and Lopez (13). For total ATPase reaction mixture, 0.8 mL of imidazole buffer (0.135 mm) with 100 mM NaCl, 20 mM KCl and 5 mM MgCl, were taken in each test tube and 0.1 mL of enzyme (a quantity that depends on the protein mg/h of enzyme source) was added and stirred. To this mixture, 0.1 mL of toxin at each of four concentrations (250 µg, 500 µg, 750 µg, and 1,000 µg) was added immediately by using micropipettes.

For the Mg^{2+}-ATPase reaction mixture, 0.07 mL of obtain (1 mM) was added as inhibitor for Na^+-K^+-ATPase in addition to the above mixture. Triple distilled water (0.1 mL) was added to the total ATPase reaction mixture and 0.03 mL of triple distilled water was added to Mg^{2+}-ATPase mixture to bring the reaction mixture to a total volume of 1.05 mL.

The reaction was started by adding 50 µL of ATP substrate (4.5mM) in each tube. All the tubes were gently shaken and incubated at 37°C for 30 minutes in a water bath. By adding 0.5 mL of 10% TCA the reaction was stopped and the contents of all tubes were centrifuged and the supernatants removed. To this supernatant, 0.3 mL of 0.1 N sodium acetate solution followed by 0.4 mL of ammonium molybdate (1%) and H_2SO_4 (0.05 N) solution were added to each tube. The color development was measured at 800 nm in a spectrophotometer after 15 minutes. The control experiment was run simultaneously with 100 µL of triple distilled water instead of toxins.

Calculation

$$\text{ATP} = \frac{\text{Absorbance of the sample x Amount of phosphate present in the standard x 60}}{\text{Absorbance of the standard x Amount of protein in the sample x Incubation time}}$$

Amount of phosphate present in the standard = 3 µg/mL

Na^+-K^+-ATPase activity = Total ATPase activity – Mg^{++}-ATPase activity

In Vitro Evaluation of Toxin Effect on the Mouse Brain AchE Enzyme

Preparation of enzyme source

The mouse brain AchE enzyme activity was assessed according to Ellman *et al.* (14). The brain isolated from the male albino mouse weighing 20

± 2 g was homogenized with 0.25M (8.55 g in 100 mL) ice cold sucrose solution and 2% (w/v) tissue homogenate was prepared in the same sucrose solution and stored in the freezer as the enzyme source.

Three milliliters of phosphate buffer (pH 8.0) was placed in each tube, to which 0.1 mL of enzyme source (2% w/v homogenate) was added and stirred. Then 100 µL of 0.01 M DTNB (0.5-5- dithiobis-2-nitrobenzoic acid) was added and the initial color was measured spectrophotometrically at 412 nm. The test solutions of toxin (100 µL) at each of four concentrations (250 µg, 500 µg, 750 µg and 1000 µg) were added. Control experiment was run simultaneously with 100 µL of triple distilled water instead of toxins.

To start the reaction, 20 µl of acetyl thiocholine iodide (ATCI) (0.075 M) was added to each tube as substrate and then the reaction was allowed to continue for 15 minutes at room temperature. The color development was measured as the final spectrophotometric reading at 412 nm. Duplicate was maintained for every experiment.

Calculation

$$\text{ATP} = \frac{\text{Absorbance of sample x Amount of Ach in present in the standard x 60}}{\text{Absorbance of standard x Amount of protein in sample x Incubation time in minutes}}$$

Where:
Absorbance = Final reading – Initial reading
Amount of Ach in the standard = 4.43 mg
Incubation time = 15 minutes

Anticancer Activity

Cell Line Toxicity Assay

The mouse muscle cell line (L929) and leukemia cell line (P388) were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were sub-cultured in a fresh flask containing Eagle's minimal essential medium (EMEM) with 10% fetal bovine serum. The cells were rinsed in serum free medium and trypsin (0.1%) was added to remove the cells from the substrate. When the monolayer had detached from the flask, the trypsin activity was stopped by adding serum-containing medium (serum contains the trypsin inhibitor α-1 antitrypsine). The cells were counted and the flask concentration was adjusted to 10^4 to 10^5 cells/mL and seeded onto a 96-well microtiter plate at the rate of 200 µL/well and incubated at 37°C.

When the wells had reached 80% confluence, they were used for cytotoxicity assay with toxins of the target species. The crude samples were diluted in EMEM at the rate of 1 mg/mL and serial 10-fold dilutions were made in sterile tubes with 2% fetal calf serum. The dilutions without the toxins served as the control. The plates were incubated at 37°C for 48 hours. The cells were observed under an inverted microscope for changes in their morphology.

After 48 hours of incubation, the toxin-treated plates were taken for the MTT (3, [4, 5-dimethylthiazole-2, 4]-2-5-diphenyl tetrazolium bromide) cell-viability test. MTT stock solution was prepared (1 mg/mL) and filtrates were sterilized in a 0.22-µm membrane filter. Fifty microliters of MTT stock solution was added to each well. The plates were incubated in a humidified incubator at 37°C for 4 hours. Then, the medium was replaced with 200 µL of DMSO and incubated for ten minutes on a microplate shaker to dissolve the formazan crystals of MTT. Twenty-five microliters of Sorensen's glycine buffer (0.1M glycine and 0.1M NaCl) was added to each well. The plates were then read at 570 nm against a reference of 690 nm in an ELISA reader. Plates were read within 30 minutes after the addition of DMSO. Samples were tested thrice for confirmation.

RESULTS

Identification of TTX Producing Bacteria (MIS)

The bacteria were isolated by the streak plate method. The pure cultures of three different strains were identified by gas chromatography. The bacterial species identified were *Kytococcus sedentarius*, *Cellulomonas fimi* and *Bacillus lentimorbus* respectively from the skin, intestine and liver.

Hemolytic Assay

The crude microbial extract from different parts of the pufferfish *Arothron hispidus* showed hemolytic activity on chicken and human blood in different blood groups (A, B, AB and O). On chicken blood, *Kytococcus sedentarius* from

the pufferfish skin (S), *Cellulomonas fimi* from the intestine (I) and *Bacillus* sp. from the liver (L) showed activity up to 32 HU. *Kytococcus sedentarius* (S) on human blood groups A, B, AB and O showed activity up to 2 HU, 32 HU, 16 HU and 32 HU respectively. *Bacillus* sp. (L) on human blood groups A, B, AB and O were up to 16 HU, 16 HU, 4 HU and 16 HU, respectively. *Cellulomonas fimi* (I) on human blood group A, B, AB and O showed activity up to 16 HU, 32 HU, 4 HU and 4 HU, respectively.

Mice Bioassay for Lethality

Crude pufferfish extract, when intraperitoneally injected into male albino mice at doses of 0.25, 0.50, 0.75 and 1.0 mL, showed symptoms of toxicity (Table 1).

Behavioral Changes in Mice

Lying on belly with forelimbs spread wide, running around the cage in an excited manner, escape reaction, prolonged palpitation, closed eyes, grooming, shivering of forelimbs, loss of balance, opaque eyes, squeaking, tonic convulsions, gasping for breath, arching of body backwards, paralysis, micturiction, flexing of muscles, prodding (insensitive to stimuli), diarrhea, lethargy, dragging of hind limbs, rolling of tail, foaming from mouth and exophthalmia were the common alterations observed upon envenomation on samples L, I and S (Table 1).

Neuromodulatory Activity

In Vitro Effect of Toxin on Mouse Brain ATPase Enzyme

Effect of ATPase enzyme was evaluated by using a single concentration of crude microbial extract to observe the modulatory activity on *Kytococcus sedentarius* (S), *Cellulomonas fimi* (I) and *Bacillus* sp. (L) shown in Figure 1.

Effect of Toxin on Mouse Brain Total ATPase Activity

The respective positive modulations produced by *Arothron hispidus* were: up to 1300% in *Kytococcus sedentarius*, up to 620% in *Cellulomonas fimi* and up to 40% in *Bacillus* sp.

In Vitro Effect of Toxin on Mouse Brain Mg^{2+}-ATPase Activity

Positive modulation by *Arothron hispidus* was observed in *Kytococcus sedentarius*, *Cellulomonas*

Table 1. Toxicity of various pufferfish samples at 5.0 mg/mL intraperitoneally injected into male albino mice (20 g ± 2)

Sample number	Extract	Injected volume (mL)	Symptoms
1	Liver (L)	0.25	No symptom is observed, after 5 min. excessive defecation
		0.50	Palpitation, excessive defecation, **not lethal**
		0.75	Palpitation, escape reaction, **not lethal**
		1.0	Palpitation, excessive urination (micturiction), **not lethal**
2	Intestine (I)	0.25	No symptom is observed, after 5 min. excessive defecation
		0.50	Palpitation, **not lethal**
		0.75	Palpitation, escape reaction, **not lethal**
		1.0	Palpitation, excessive urination (micturiction), **not lethal**
3	Skin (S)	0.25	Palpitation, **not lethal**
		0.50	Palpitation, escape reaction, **not lethal**
		0.75	Palpitation, excessive urination (micturiction), **not lethal**
		1.0	Palpitation, escape reaction after 5 min., dragging of hind limbs

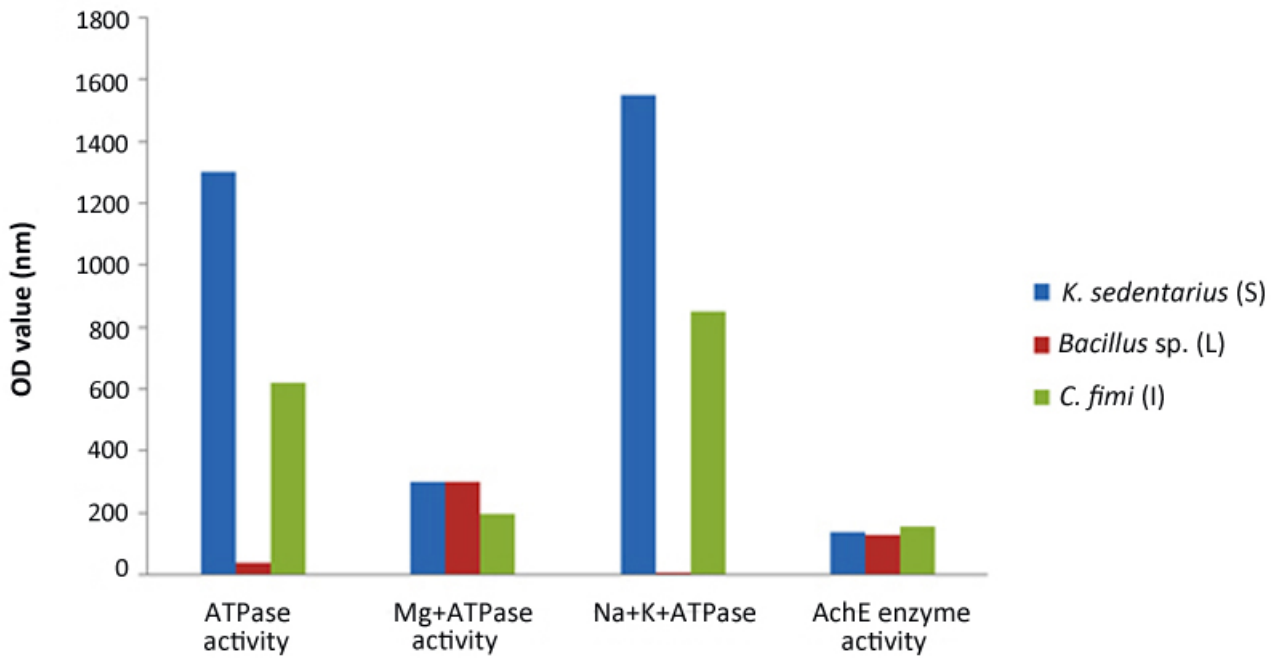

Figure 1. *In vitro* effect of toxin on mouse brain total ATPase, Mg^{+}-ATPase, Na^{+}-K^{+}-ATPase and AchE enzyme activity.

fimi and *Bacillus* sp., up to 300.1, 300 and 200%, respectively.

In Vitro Effect of Toxin on Mouse Brain Na^{+}-K^{+}-ATPase Activity

Positive modulation by *Arothron hispidus* was verified in *Kytococcus sedentarius, Cellulomonas fimi* and *Bacillus* sp. with respective neuromodulation activities of up to 1549.98, 10.0 and 849.98%.

In Vitro Effect of Toxin on Mouse Brain AchE Enzyme

Arothron hispidus presented positive modulations in *Kytococcus sedentarius*, *Cellulomonas fimi* and *Bacillus* sp. of up to 140.55, 128.42 and 158.69% respectively.

Anticancer Activity

The viability of mouse muscle cell line (L929) and leukemia cell line (P388) were adversely affected upon adding the crude microbial extracts. The toxicity symptoms shown by the cells were rounding, lysis and detachment from the substratum.

The growth inhibition levels of the L929 cell line in the crude microbial extract from *Arothron hispidus* ranged between a maximum of 94.43% at the lowest dilution and a minimum of 84.03% at the highest dilution in the case of *Bacillus* sp. In the case of the crude microbial extract of the samples of *Cellulomonas fimi* (I) and *Kytococcus sedentarius* (S) the inhibition levels ranged from 75.82 to 88.47%.

Concerning the leukemia cell line P388, the crude microbial extract of *Arothron hispidus,* inhibited the growth at the lowest dilution of 86.16% and at the highest dilution by 77.25% in *Bacillus* sp. The crude microbial extract of *Cellulomonas fimi* (I) and *Kytococcus sedentarius* (S) inhibited growth by 64.75 and 76.31%, respectively, at the lowest dilution of toxin and by 51.32 and 67.90% at the highest dilution. However, in both L929 and P388 cell lines, the inhibition was dose-dependent, that is, higher inhibition at lower dilutions and lower inhibition at higher dilutions of the crude extract of these pufferfish samples as shown in Figures 2, 3 and 4.

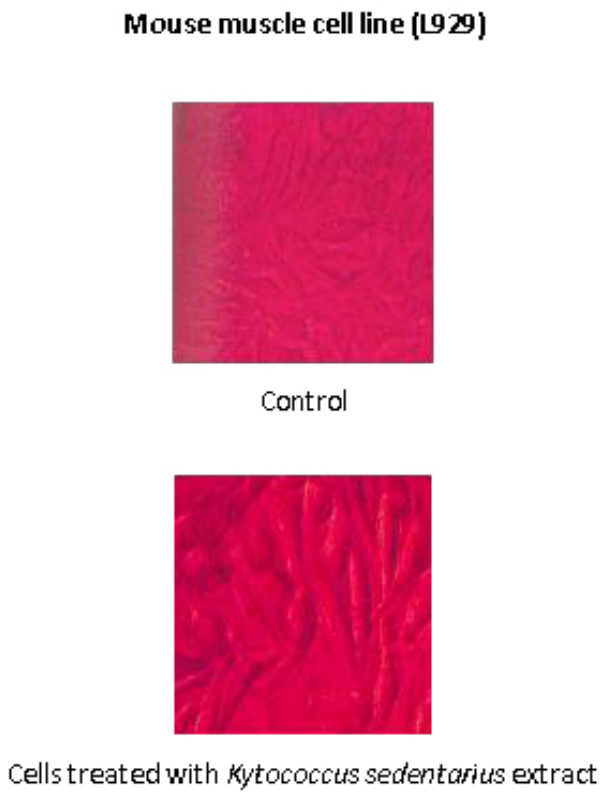

Figure 2. Growth inhibitory activity of *Kytococcus sedentarius* bacterial crude extract obtained from *Arothron hispidus* skin against two cell lines.

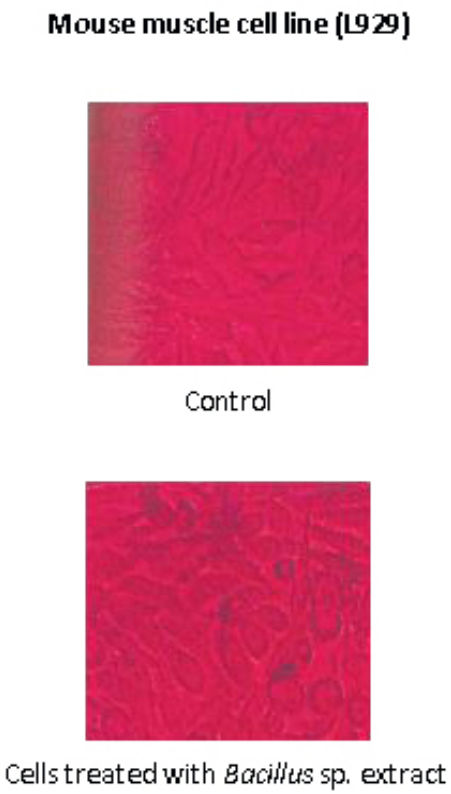

Figure 3. Growth inhibitory activity of *Bacillus* sp. bacterial crude extract obtained from *Arothron hispidus* liver against two cell lines.

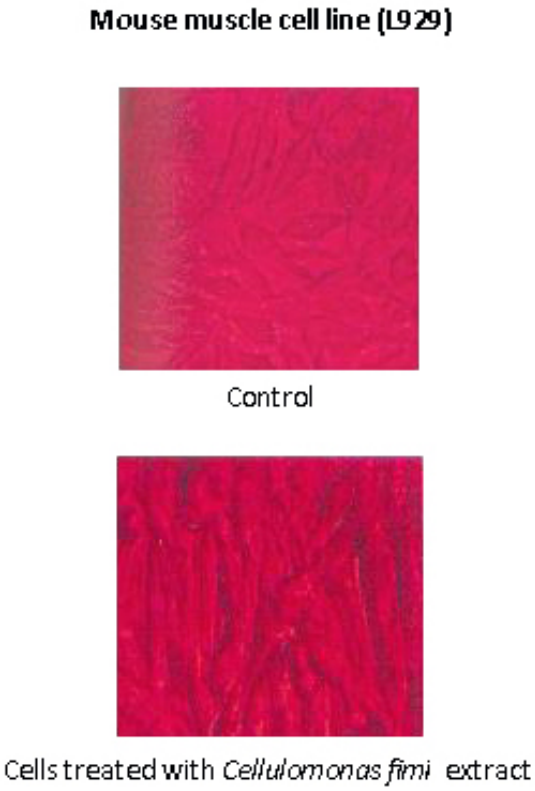

Figure 4. Growth inhibitory activity of *Cellulomonas fimi* bacterial crude extract from *Arothron hispidus* intestine against two cell lines.

DISCUSSION

In the present study, three TTX-producing bacteria (*Kytococcus sedentarius*, *Cellulomonas fimi* and *Bacillus* species) were isolated from the pufferfish *Arothron hispidus* collected at Parangipettai, southeast India, by using the Microbial Identification System (MIS). The *Bacillus* species are dominant in the liver and intestine of *Arothron hispidus* as reported in an earlier study (15). This species and *Kytococcus sedentarius, Cellulomonas fimi* were examined for their ability to produce TTX by hemolytic activity and mouse bioassay. The TTX-producing bacteria (*Vibrio* sp., *Pseudomonuas* sp., *Pasteurella* sp., *Aeromonas* sp. and *Plesiomonas* sp.) could cause disease in marine animals and were isolated from digestive glands and muscles of the Taiwanese gastropod, *Niotha clathrata,* the intestines of the wild Korian pufferfish, *Takifugu vermicularis*, and the spine catch apparatus of the Caribbean sea urchin, *Meoma ventricosa* (15-19).

The current research on the evolution of tetrodotoxin supports the notion of a symbiosis between bacteria and tetrodotoxin-resistant animals. TTX can be isolated rapidly from diverse animal species which were totally unrelated phylogenetically to each other (20). Later in 1995-2000, few studies have been conducted to investigate TTX-bearing animals and TTX-producing bacteria (8).

Chicken blood hemolysis showed activity on all three species while the human blood showed intense activity on *Kytococcus sedentarius* (S). Certain TTX toxins are host-specific and provoke cytolysis against a narrow range of target cells, while *E. coli* hemolysin is toxic to a wide range of mammalian cell types (21-25).

Toxicity symptoms were noted in the present study. The common behavioral changes observed upon envenomation of samples were: tested mice lying on the belly with forelimbs spread wide, frenzied running around the cage, escape reaction, prolonged palpitation, closed eyes, grooming, shivering of forelimbs, loss of balance, opaque eyes, squeaking, tonic convulsions, gasping for breath, arching of body backwards, paralysis, micturition, flexing of muscles, prodding (insensitive to stimuli), diarrhea, lethargy, dragging of hind limbs, rolling of tail, foaming from the mouth and exophthalmia.

TTX itself is a potent neurotoxin that blocks sodium channels with great specificity and can produce rapid and violent death in humans and other animals even in small quantities (26). All three TTX-producing bacteria had either a stimulatory or inhibitory effect on the neuronal system of mice. The ATPase enzyme system is widely accepted as deriving a part of its energy from ATP hydrolysis for active transport of Na^+-K^+-ATPase. Present findings showed that the toxins affected the neuronal system of mice whereas *Kytococcus sedentarius* derived from pufferfish skin samples presented high modulatory activity. The pharmacological activity of tetrodotoxin on various animals, their organs and tissues has been extensively studied, and it was found that tetrodotoxin strongly inhibits nerve conduction (27). Tetrodotoxin inhibitory effects on Na^+-K^+-ATPase activity are well documented, as in the case of the marine invertebrate toxin paramyocine from the squid *Todarodes pacificus* (28-30). This corroborates the study by Fatma (31), who demonstrated that TTX has a great ability to bind to the transmembrane glycoprotein that forms the Na^+ channel which resulted in blocking. The AchE activity of *Bacillus* sp (L) was proven to be highly modulatory.

The results of the present study showed an antitumor effect of TTX against leukemia cell line (P388). The inhibitory activity of *Bacillus* sp. in a mouse cell line (L929) was in agreement with that obtained from treatment with masked pufferfish, green tea, *Indigofera* plant extract, *Nigella sativa* seeds and *Bauhinia racemosa* plant (32-36). Despite the fact that TTX is well documented as a pain relief agent because of its ability to block the Na^+ channel in the nervous system, it has been demonstrated that the action mechanism of TTX on Ehrlich ascite carcinoma is probably not different from that on other cells in the nervous system. It plays an important role in binding to p-glycoprotein of the Na^+ channels, blocking it and preventing Na^+ influx into the cells. This action, in turn, prevents the carcinoma cells from getting sufficient Na^+ ions for the requirements of various intracellular functions, and above all to maintain the normal charge distribution across the cell membrane, a process necessary to maintain cellular integrity. As a consequence, the proliferation and invasiveness of such cells are suppressed. The results clearly demonstrated the effectiveness of TTX on the development of mouse cell line. There was a considerable reduction in the number of cells during the first six days of the experiment.

CONCLUSION

The present study clearly indicates the toxic nature of pufferfish due to associated bacteria. The extracts show behavioral changes that are not lethal to mice upon intraperitoneal administration. Potent hemolytic and cytotoxic activities are detected. These compounds are worth studying for possible antineoplastic substances. The toxicity pathway was shown to be the modulation of ATPase and AChE enzymes, rendering the TTX to be CNS-active. Thus, it can be concluded that toxins from the bacterial species stand out as excellent candidates for further research on the development of their potential biomedical compounds. TTX-producing bacteria from *Arothron hispidus* also hold promise for the development of effective antitumor compounds.

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CONFLICTS OF INTEREST

There is no conflict.

FINANCIAL SOURCE

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Institutional Ethics Committee (regulation number 160/1999/CPCSEA/11.01.2008) of the Rajah Muthiah Medical College, Annamalai University, India.

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