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Studies on antimicrobial activity and brine shrimp lethality of crude samples of six different species of puffer fishes

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ABSTRACT

Objective: To evaluate the antimicrobial activity and brine shrimp lethality activity of six different species of puffer fishes, including *Cyclichthys orbicularis, Diodon holocanthus, Canthigaster solandri, Arthron hispidus, A. inermis* and *Lagocephalua inermis* (*L. inermis*).
Methodology: The puffer fishes were collected from Annangkovil Fish Landing Centre (Lattitude 11°30.47' N; Longitude 79°47.02' E), Parangipettai, Southeast Coast of India during summer season because of availability. Fresh tissue samples were collected from the clearly washed specimens, extracted with methanol at 37 ˚C for 3 days and filtered through Whatman No. 1 filter paper. The solvents such as methanol and ethanol were concentrated by using rotary evaporator under reduced pressure. The dark brown gummy mass was stored at 4 ˚C for further analysis. Prepared crude samples were analysed with human pathogens to assess the antibacterial activity and this was carried out by using standard disc diffusion method. The brine shrimp lethality was calculated as the percentage of mortality which was firstly calculated by dividing the number of dead larvae by the total number and then multiplied to 100%.
Results: The antibacterial activity of crude extract of puffer fishes were exhibited against 10 different human bacterial pathogens. Among the ten human pathogens, *Arthron hispidus* showed maximum zone of inhibition (8 mm) against *Staphylococcus aureus* while *L. inermis* showed minimum activity (1 mm) against *Proteus mirabilis* and no zone of inhibition was observed against *Staphylococcus aureus*. Brine shrimp lethality was examined with six puffer fish extracts. *Cyclichthys orbicularis* showed maximum mortalities as 100% and *L. inermis* showed minimum mortalities as 70% at a concentration of 500 μg/mL.
Conclusion: In conclusion, the study showed the preliminary investigation of crude extracts of puffer fishes about the prominent activity against human bacterial pathogens. The extracts had a good cytotoxic potential against brine shrimp *Artemina salina*.

1. Introduction

The ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity. The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural and chemical features not found in terrestrial natural products. The richness of diversity offers a great opportunity for the discovery of new bioactive compounds. The number of natural products isolated from marine organisms increases rapidly, and now exceeds with hundreds of new compounds being discovered every year[1,2]. As the evolution progresses, microbes have evolved themselves and became resistant to available antimicrobial drugs. Thus, modern technologies have opened vast areas of research for the extraction of biomedical compounds from ocean and seas to treat the deadly diseases.

Many classes of natural products from marine sources exhibiting antitumor, anti-leukaemia, antibacterial and antiviral activities have been reported worldwide. Marine fishes are able to produce bioactive compounds on both epidermal mucus and the whole body that contain antibacterial activity to protect them from dangerous pathogens. The broad usage of antibiotics has led to emergence of various potent pathogens, nullifying the effect of active drug components. Hence, the world is in need of novel active biomolecules in order to combat the emerging and re-emerging pathogens hindering aquatic and human health status. Fishes form a diverse group of animals, highly specialized for their aquatic existence and comprising almost half the number of vertebrate species in existence today. Fishes are in intimate contact with their environment containing very high concentration of bacteria and

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viruses, many of which are saprophytic while some are pathogenic and both are capable of digesting and degrading the fish tissues. However, under normal conditions, the fish maintains a healthy state by defending itself against these potential invaders by a complex system of innate and adaptive defence mechanisms[3]. So all fishes maintain some secondary metabolites for defence mechanism. However, in fishes, the specific immune mechanisms are slow and limited by temperature which can constrain their metabolism. Puffer fishes are able to produce tetra-toxin with associate microbes on the mucus of their body. Based on the above information, the present study aims to evaluate antimicrobial activity, and brine shrimp lethality with six different species of puffer fishes.

2. Materials and methods

2.1. Sample collection and preservation

The marine puffer fishes *Cyclichthys orbicularis* (*C. orbicularis*), *Diodon holocanthus* (*D. holocanthus*), *Canthigaster solandri* (*C. solandri*), *Arthron hispidus* (*A. hispidus*), *A. inermis* and *Lagocephalua inermis* (*L. inermis*) were collected from Annangkovil Fish Landing Centre (Lattitude 11°30.47' N; Longitude 79°47.02' E), Parangipettai, Tamilnadu, Southeast coast of India during summer. The collected poisonous puffer fishes were transported to the laboratory carefully at room temperature.

2.2. Collection of crude sample

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

2.3. Microbial pathogens

All bacterial strains such as *Escherichia coli* (*E. coli*), *Klebsiella oxytoca* (*K. oxytoca*), *Klebsiella pneumonia* (*K. pneumonia*), *Proteus mirabilis* (*P. mirabilis*), *Salmonella paratyphi* (*S. paratyphi*), *Salmonella typhi* (*S. typhi*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogens* (*S. pyogens*), *Vibrio cholera* (*V. cholera*), and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) were obtained from Rajah Muthaih Medical College, Annamalai University, Annamalai Nagar, Chidambaram, Tamilnadu, India.

2.4. Antibacterial activity

Prepared crude samples were analysed with human pathogens to assess the antibacterial activity and this was carried out by using standard disc diffusion method. Gurudeeban *et al.* has described the method of evaluating antibacterial activity. The bacterial strains were subcultured and swabbed on surface of the Muller Hinton agar. The prepared antibiotic discs (Whatman No. 1 filter paper with 6 mm diameter) were impregnated with 50 µL of six crude extracts and placed on the surface of the plate. Control discs were placed with respective solvents and tetracycline (30 µL/mL) was used as a positive control. All the plates were incubated at 37 ˚C for 24 h and the zones of inhibition were measured. The susceptibility of the test organisms were determined by radius of the inhibition zone around each disc. All extracts were tested with triplicate at a concentration of 30 mg per disc[4].

2.5. Brine shrimp lethality assay

Brine shrimp eggs were obtained from CP aquaculture, Nellore, Andrapradesh, India. The artificial sea water was prepared by dissolving 38 g of sea salt in 1 L of disc. Water for hatching the shrimp eggs was put into a plastic container which contained dark and light areas separately. Two days were needed for the shrimp to hatch and mature as larva. Briefly, 4 mL of the artificial sea water and 10 brine shrimps were introduced into each test tube; and the total volume was made into 5 mL. The number of surviving shrimps were counted and recorded after 24 h. Using profit analysis, the lethality concentration at 100, 200, 300, 400, and 500 µg/mL [median lethal concentration (LC_{50})] were assessed at 95% confidence intervals. LC_{50} value of less than 1 000 µg/mL is toxic while LC_{50} value of greater than 1 000 µg/mL is non-toxic. The percentage of mortality was firstly calculated by dividing the number of dead larvae by the total number and then multiplied to 100%.

2.6. Statistical analysis

Origin (6.0) software was used for statistical analysis.

3. Results

The present study has exhibited the antimicrobial activity of crude methonol extracts of six different species of puffer fishes against ten species human bacterials. The six different species of puffer fishes showed variations in the activity against pathogens (Figure.1). The puffer fish *C. orbicularis* showed maximum activity (6 mm) against *Proteus mirabilis* and minimum activity against *K. oxytoca, V. cholera* and *V. parahaemolyticus* (3 mm). Whereas *D. holocanthus* showed maximum activity (6 mm) against *E. coli* and minimum activity against *P. mirabilis* (1 mm).

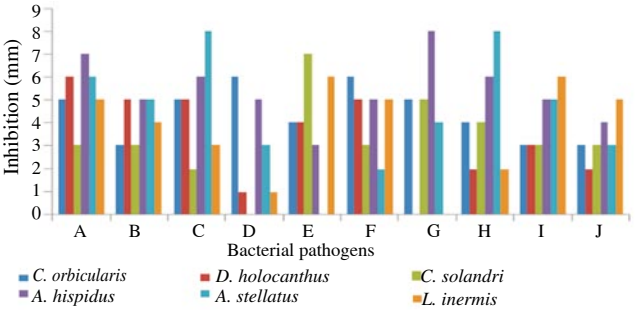

Figure 1. Six different buffer fishes extracts show the variations in the activity against ten different human pathogens.

A: *E. coli*; B: *K. oxytoca*; C: *K. pneumoniae*; D: *P. mirabilis*; E: *S. paratyphi*; F: *S. typhi*; G: *S. aureus*; H: *S. pyogens*; I: *V. cholerae*; J: *V. parahaemolyticus*.

The *C. solandri* showed maximum activity (7 mm) against *S. paratyphi* and minimum activity (2 mm) against *K. pneumoniae,* and activity was nil against *P. mirabilis*. The *A. hispidus* showed maximum activity (8 mm) against *S. aureus* and minimum activity (3 mm) against *S. paratyphi*. The *A. Stellatus* showed maximum activity (8 mm) against *S. pyogens* and minimum activity (2 mm) against *S. typhi* and activity was zero against *S. paratyphi*. The *L. inermis* showed maximum activity (6 mm) against *S. paratyphi* and *V. cholerae* and minimum activity (1 mm) showed against *P. mirabilis* and the activity was nil against *S. aureus*.

The brine shrimp lethality caused by different concentrations (100, 200, 300, 400, and 500 µg/mL) of crude sample of six different species of puffer fishes, *C. orbicularis*, *D. holocanthus*, *C. solandri*, *A. hispidus*, *A. inermis* and *L. inermis* was shown in Figure 2. The degree of lethality was directly proportional to the concentration of the sample. The low cost and commercial availability of shrimp eggs make brine shrimp lethality assay a powerful model. Accordingly,

in the present study, *C. orbicularis* showed maximum mortality as 100% at a concentration of 400 and 500 µg/mL and minimum mortality was observed as 30% mortalities at a concentration of 100 µg/mL.

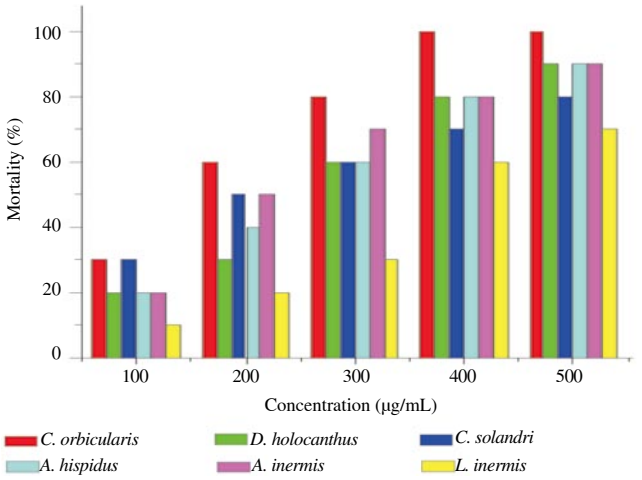

Figure 2. The brine shrimp lethality caused by different concentrations (100, 200, 300, 400 and 500 µg/mL) of crude sample of six different species of buffer fishes.

D. holocanthus showed maximum mortality as 90% at a concentration of 500 µg/mL. *C. solandri* showed maximum mortality as 80% at a concentration of 500 µg/mL and minimum mortality was showed as 30% at a concentration of 100 µg/mL. *A. hispidus* showed maximum mortality as 90% at a concentration of 500 µg/mL and minimum mortality was showed as 20% at a concentration of 100 µg/mL. *A. inermis* showed maximum mortality as 90% at a concentration of 500 µg/mL and minimum mortality was showed as 20% at a concentration of 100 µg/mL. *L. inermis* showed maximum mortality as 70% at a concentration of 500 µg/mL and minimum mortality was showed as 10% at a concentration of 100 µg/mL. (Methodology part mentioned the probit analysis from brine shrimp mortality but the result did not show the LC_{50} and LC_{90} values, please revise).

4. Discussion

The rationale of probing for drugs from marine environment is the fact that marine plants and animals have adapted to all sorts of marine environments and these beings are constantly under marvellous selection pressure including space competition, predation, surface fouling and reproduction. Most of the marine creatures have been found to possess antimicrobial activity against human bacterial pathogens as well as fungi and viruses, and most of the antibacterial agents have derived from marine sources. In addition, the present study also helps identify the antibacterial and brine shrimp lethality from six species of puffer fishes against 10 different types of human bacterial pathogens.

Antimicrobial peptides in marine organisms are existing candidates for the development of new antibacterial compounds, due to their broad activity spectrum and difficulty for bacteria to develop resistance to them[5]. Rethna Priya *et al.* discussed that pip fishes showed different antimicrobial activity against a range of both Gram-positive and -negative pathogenic bacterial strains[6] and Phongpaichit *et al.* fraction exhibited low inhibition activity against *S. aureus* and *E. coli* with narrow inhibition zones of 6.8-10.8 mm and minimal inhibitory concentration values of 5-10 mg/mL. Previous study has reported that the methonolic extract of puffer fish *A. calamus* had no activity against *Enterococus* sp. and *P. aeruginosa*[7] but in the case of the present study, it demonstrated that the inhibition zone of the human bacterial pathogens were 1-8 mm.

Mohana Priya *et al.* reached to the result that the crude tissue extracts of *A. hispidus* were screened against seven human pathogenic bacteria for testing their antibacterial activities and the inhibition zones of the extracts were compared with standard ampicillin for bacterial culture. Therefore, they observed maximum zones against *E. coli* in the skin extracts of *A. hispidus* and the minimum zones was observed against *Proteus vulgaris* in the liver extracts[8]. But the present study exploring six different species of puffer fishes against 10 different human bacterial pathogens showed different inhibitions zones. Among the six samples, *A. hispidus* showed maximum activity (8 mm) against *S. aureus* and *L. inermis* showed minimum activity (1 mm) against *P. mirabilis* and the activity was nil against *S. aureus*.

Mohan *et al.* claimed that maximum mortality was recorded as 80% at a concentration of 100 µg/mL[9] but in the present study, among the six puffer fishes extracts, *C. orbicularis* showed maximum mortality as 100% and *L. inermis* showed minimum mortality as 70% at a concentration of 500 µg/mL; *C. solandri* showed maximum mortalities as 30% and minimum mortality of *L. inermis* crude sample was observed as 10% at a concentration of 100 µg/mL.

The present study concluded that the crude extracts of puffer fishes had pharmacological effect against pathogens, particularly human pathogens. And the extracts have a good cytotoxic potential against brine shrimp *Artemina salina.*

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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