Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2016; 8(5); 312-319

ISSN: 0975-5160

Research Article

Antimicrobial and Larvicidal Activities of the Tissue Extracts of Oblong Blowfish (*Takifugu oblongus)* from South - East Coast of India

Indumathi S M, Manigandan V, Khora S S*

Department of Integrative Biology, School of Biosciences and Technology, VIT University, Vellore - 632014, Tamil Nadu, India.

Available Online: 25th October, 2016

ABSTRACT

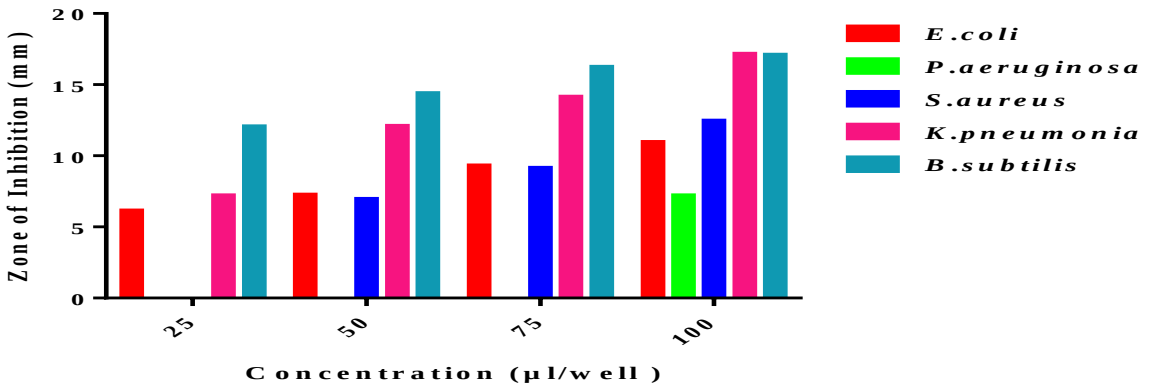
Discovery of Bioactive compounds from marine sources especially fishes have always been of great importance since several decades and it is much more essential to identify novel natural products which might possess significant properties to treat various diseases. The main objective of this study is to evaluate the *in vitro* antibacterial, antifungal and larvicidal activities of various crude tissue extracts of the puffer fish *Takifugu oblongus* collected from the South – East Coast of India. The amount of protein present in each tissue was determined by spectrophotometric methods. The *in vitro* antibacterial and antifungal activities of the tissue extracts against various bacterial and fungal pathogens were investigated by well diffusion method. Larvicidal activity of the tissue extracts was assessed by analyzing the mortality rate of all tested larvae. Muscle extract showed highest protein content (340 µg/ml), whilst gonads extract showed least protein concentration (94 µg/ml) in the puffer fish. The measured zones of inhibition of various pathogens by different tissue extracts proved that gonads extract possessed highly commendable antibacterial activity against most bacterial strains. And muscle extract recorded lowest antibacterial activity. No antifungal activity was observed with all the tissue extracts. Though all the tissue extracts produced significant larvicidal activities, maximum activity was noticed with skin extract, whereas muscle extract showed the least. The crude tissue extracts of the puffer fish *Takifugu oblongus* showed noteworthy bioactivities and so further studies may be recommended to identify the natural bioactive compounds, so that they could be promising candidates for drug development.

Keywords: *Takifugu oblongus*, Antibacterial, Antifungal, Larvicidal, Bioactive Compounds.

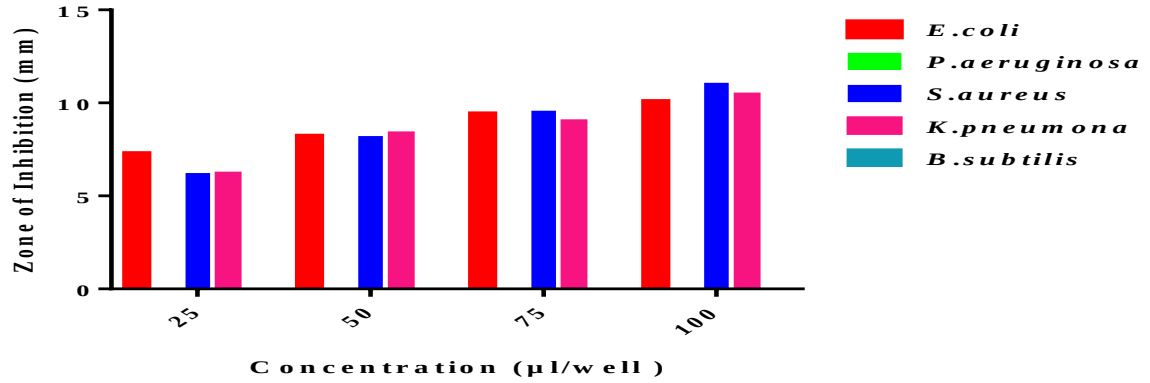
INTRODUCTION

The marine biota is the largest source for novel discovery of natural products or bio similarities such as pharmacological metabolites and medicines. There has been an extensive research showing that vast bioactive substances were identified and characterized from marine organisms, indeed several of them showed promising results to treat human and animal diseases[1,2]. For the past two decades, the major obstacle to treat microbial infections is the emergence of microbial resistance towards majority synthetic compounds and also evolution of multiple mechanisms towards microbial resistance as well, confined the use of antimicrobial compounds[3,4]. The urge of natural antimicrobial therapeutics discovery and development is always in demand for pharmaceutical industries, and its vital importance is to escape the risk of resistance development by pathogenic microorganism[5]. Till date, the secondary metabolites derived from the various marine organisms possess antimicrobial and anticancer properties, while some of them are under clinical studies[6,7]. Didemnin B from the tunicate *Tridemnum solidum* was the first marine natural compound to enter clinical trials as an anticancer agent, but no longer continued owing to its side effects[7,8]. The invertebrates of marine biota like sponges, coelenterates and tunicates account the large number of pharmacological compounds[9], whereas fish is the earliest and the largest class vertebrate in marine biota with its innate immune system being considered as the predominant mechanism for host defense and this includes excretion of antimicrobial peptides, polypeptides, non-classical complement activation, cytokine release, inflammation and phagocytosis[10-13]. Precisely, fishes evolved several innate immune mechanisms to defend microbial infection[11]. On the other hand, fishes hold the credit of possessing rich protein sources. These marine proteins are not only correlated to the intact proteins, but also to the possibility of generating bioactive peptides[14]. In recent years, different toxins derived from marine sources have been identified as having potential antimicrobial activities. Puffer fishes belonging to the family Tetraodontidae, has always been a great interest for various marine biologists for years together. The toxin found in puffer fishes is termed tetrodotoxin (TTX), which serves as specific sodium channel blocker and is highly used in neurophysiological and neuropharmacological studies[15-17]. There was some general understanding that non cultured marine puffer fish are highly toxic, acquiring this TTX by bioaccumulation via marine food chain, rather cultured puffer fishes were non toxic[18]. Awareness of the toxin

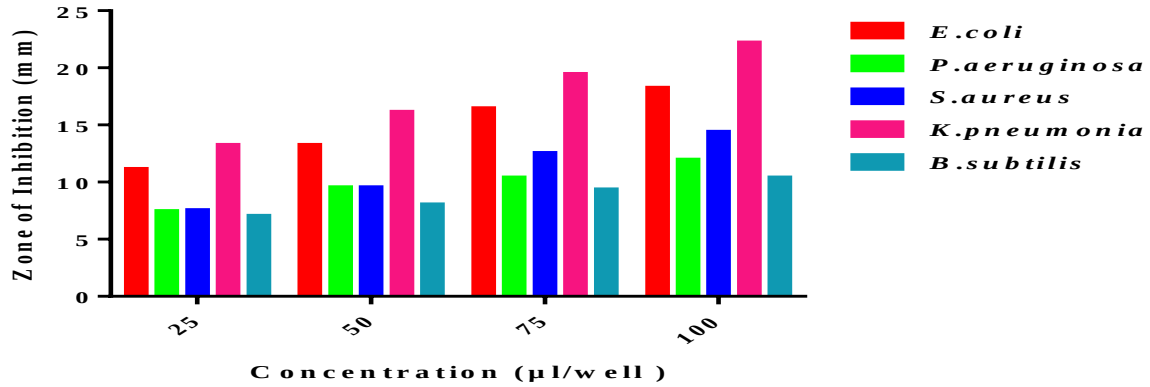
*Author for Correspondence: sskhora@vit.ac.in



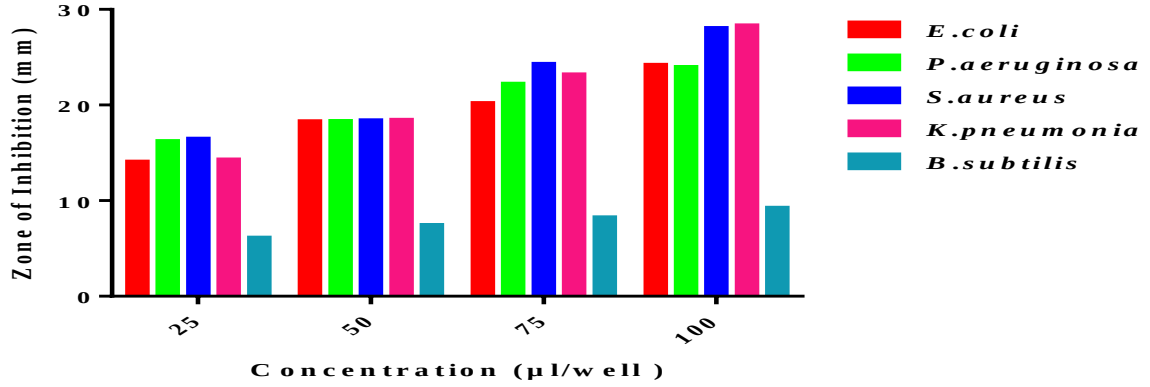
(A)



(B)



(C)



(D)

Figure 1: Antibacterial activity on various bacterial pathogens by the tissue extracts (A) Skin Extract (B) Muscle Extract (C) Liver Extract (D) Gonads Extract.

contained in puffer fishes made the ancient Chinese and Japanese to consume the poisonous organs of the fish as a general health tonic[36]. Earlier before WW II, Japanese researchers analyzed the ability of crude puffer fish extract to treat migraines and menstrual cramps[19]. Tetrodotoxin is reported to reduce the symptoms of withdrawal in heroin

addicts and serve as an analgesic to minimize pain in cancer patients[20]. The recent studies of puffer fish extracts revealed potential larvicidal activity against various mosquito larvae[21]. Apart from these, in India, studies on puffer fishes are least performed and still not well elucidated. In our study we aimed to evaluate the bioactive potentials of the puffer fish *T. oblongus* viz, antimicrobial and larvicidal activities.

MATERIALS AND METHODS

Specimen Collection

Specimens of the puffer fish *Takifugu oblongus* (Bloch, 1786) were collected from Kasimedu fishing harbour, Chennai (Lat. 13º 11' N and Long 80º 29' E) and Mandapam coastal area (Lat. 09º 28'N and Long. 79º 12' E) Ramanathapuram District, Tamil Nadu, India. A total of 67 specimens were collected which were tightly packed and transferred in Ice box to the Medical Biotechnology Lab, VIT University. And they were maintained in a deep freezer at -20 °C until use.

Preparation of toxic extract

The specimens were dissected and the tissues particularly Skin, Muscle, Liver and Gonads were excised from which the extract was prepared. 10 g of every tissue is weighed and separately homogenized in tissue homogenizer with 50 ml of 0.1% Acetic acid in water. The tissue homogenates were boiled for 10 minutes in water bath at 50 °C. They were cooled and centrifuged at 3000 rpm for 10 minutes. The supernatants were collected and stored. The same procedure was followed thrice and the supernatants collected were combined, filtered and stored[22].

Protein Estimation

The amount of protein present in the tissue extracts was analyzed through Bradford Assay[23]. Series of protein standards diluted with distilled water were prepared and the tissue samples were serially diluted as well. 100 µl of the protein standards and the diluted tissue standards were measured in different test tubes and 5 ml of coomassie blue stain is added to each tube, mixed well by vortexing. The mixture is incubated for 15 minutes in dark. The UV spectrophotometer was adjusted to a wavelength of 595 nm and OD is adjusted to 0 using blank containing distilled water. The incubated samples were read at 595 nm and OD was measured. Graph was plotted with absorbance of the standards and concentration. The concentration of proteins in the unknown samples was calculated using the extinction coefficient.

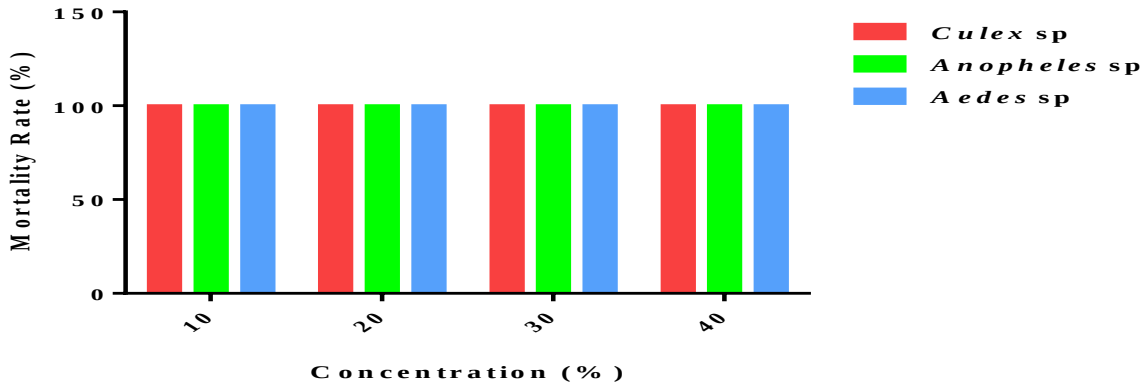
In vitro Antibacterial Activity

The antibacterial activity was determined by Well Diffusion Methods[24,25]. About 25 ml of molten Mueller Hinton Agar was poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 5 different species of pathogenic bacteria namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Bacillus subtilis* were transferred onto the plates and made lawn culture by using sterile L-rod spreader. After five minutes setting of the bacteria, the wells were made using sterile 5 mm cork borer and tissue extracts at various concentrations (i.e. 25, 50, 75 and 100 µl/well) were added to each well. Tetracycline (30 µg) served as positive control and 0.1% acetic acid served as negative control.

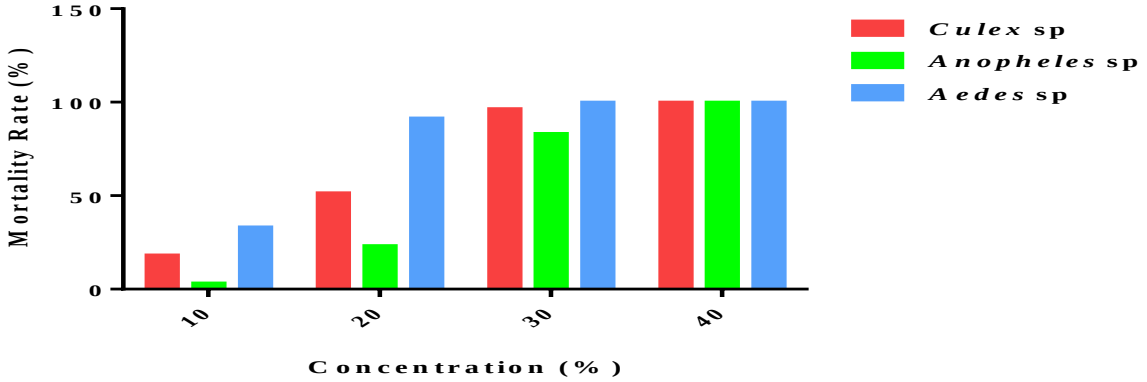
Table 1: Antibacterial activity of the tissue extracts of *T. oblongus* against bacterial pathogens.

Extracts	Organisms	Concentration (µl/well)			
		25	50	75	100
Liver					
	E. coli	11.2±0.1	13.3±0.1	16.5±0.2	18.3±0.1
	P. aeruginosa	7.5±0.1	9.6±0.1	10.45±0.15	12±0.0
	S. aureus	7.6±0.2	9.5±0.3	12.6±0.3	14.45±0.35
	K. Pneumoniae	13.3±0.1	16.2±0.2	19.5±0.3	22.25±0.15
	B. subtilis	7.1±0.1	8.1±0.1	9.4±0.2	10.45±0.25
Muscle					
	E. coli	7.25±0.05	8.2±0.2	9.4±0.4	10.05±0.05
	P. aeruginosa	-	-	-	-
	S. aureus	6.15±0.15	8.15±0.15	9.5±0.1	11±0.0
	K. pneumonia	6.2±0.2	8.35±0.35	9±0.0	10.45±0.25
	B. subtilis	-	-	-	-
Gonads					
	E. coli	14.15±0.15	18.35±0.35	20.25±0.15	24.25±0.25
	P. aeruginosa	16.3±0.1	18.4±0.4	22.3±0.1	24.05±0.05
	S. aureus	16.55±0.25	18.45±0.25	24.35±0.15	28.1±0.1
	K. Pneumoniae	14.35±0.15	18.5±0.2	23.25±0.15	28.4±0.2
	B. subtilis	6.2±0.2	7.5±0.2	8.3±0.1	9.3±0.3
Skin					
	E. coli	6.2±0.2	7.3±0.2	9.35±0.35	11±0.0
	P. aeruginosa	-	-	-	7.25±0.05
	S. aureus	-	7±0.0	9.2±0.2	12.5±0.15
	K. pneumoniae	7.25±0.25	12.15±0.15	14.2±0.2	17.2±0.0
	B. subtilis	12.1±0.1	14.45±0.05	16.3±0.0	17.15±0.15

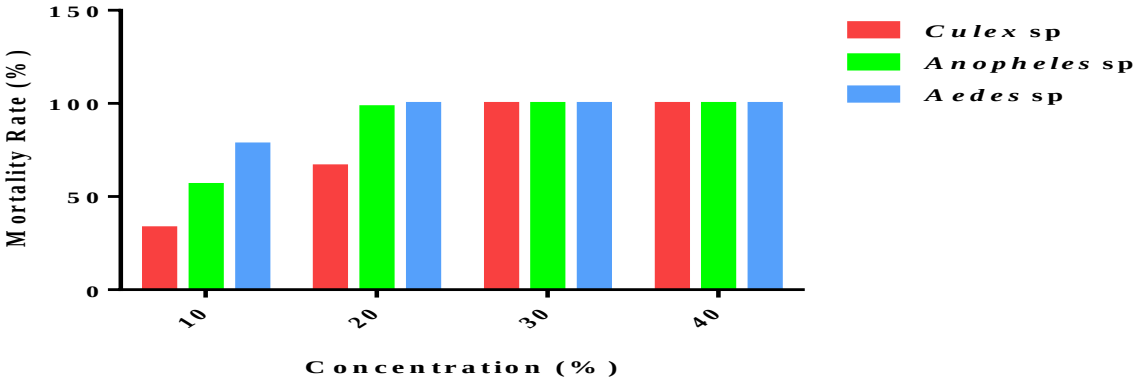
Data are expressed as mean ± S.D of 2 determinations



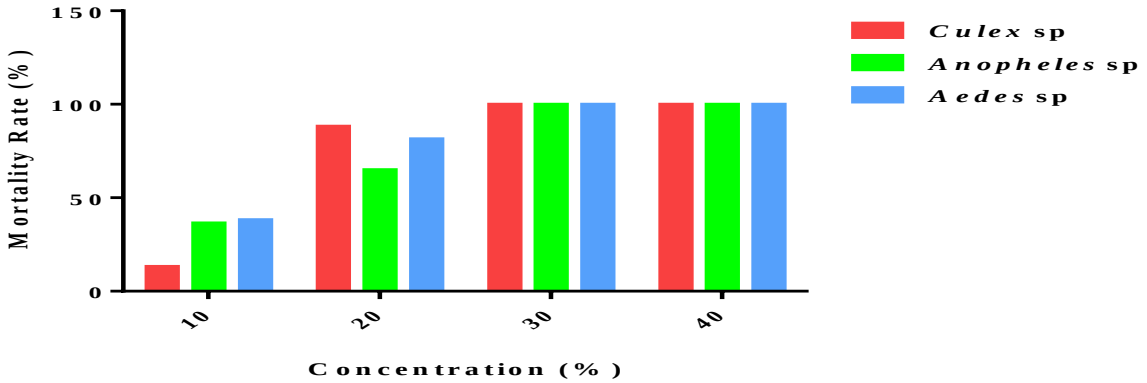
(A)



(B)



(C)



(D)

Figure 2: Larvicidal activity on various larvae by the tissue extracts. (A) Skin extract (B) Muscle Extract (C) Liver Extract (D) Gonads Extract

Table 2: Larvicidal activity of the tissue extracts of *T. oblongus* against the test larvae.

Extracts	Larvae Species	Concentration (%)			
		10	20	30	40
Liver					
	Culex sp	33.26±2.36	66.6±2.36	100±0	100±0
	Anopheles sp	56.6±4.7	98.3±2.36	100±0	100±0
	Aedes sp	78.3±9.4	100±0	100±0	100±0
Muscle					
	Culex sp	18.3±6.24	51.6±4.7	96.6±4.7	100±0
	Anopheles sp	3.3. ±1.7	23.3±8.6	83.3±4.7	100±0
	Aedes sp	33.3±7.3	91.6±2.31	100±0	100±0
Gonads					
	Culex sp	13.3±2.35	88.3±4.7	100±0	100±0
	Anopheles sp	36.5±2.36	65±4.08	100±0	100±0
	Aedes sp	38.3±4.1	81.6±4.7	100±0	100±0
Skin					
	Culex sp	100±0	100±0	100±0	100±0
	Anopheles sp	100±0	100±0	100±0	100±0
	Aedes sp	100±0	100±0	100±0	100±0

Data are expressed as mean ± S.D of 2 determinations

The plates were incubated at 37 °C in a 40 W florescent light source (~ 400 nm) for 24 h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India).

In vitro Antifungal Activity

The antifungal activity was determined by Well Diffusion Methods[24,25]. Five species of pathogenic fungi viz, *Aspergillus niger*, *Aspergillus alliaceus*, *Aspergillus tubigiensis*, *Aspergillus parasiticus globosus* and *Aspergillus flavus* were inoculated in Sabouraud Dextrose broth for overnight incubation. Fungal cultures were spread on Sabouraud Dextrose agar plates and wells were made using sterile 7 mm cork borer and tissue extracts at various concentrations (i.e. 25, 50, 75 and 100 µl/well) were added to each well. Nystatin (30 µg) was used as positive control and 0.1% acetic acid served as negative control. The plates were incubated at 25 °C to 28 °C for 48-72 h. The zone of inhibition was observed and measured using Zone Inhibitory Scale (Himedia) and the values were recorded in mm.

Larvicidal Activity

Larvicidal activity was determined by earlier studies[21,26]. The larvae were collected from stagnant rain water from the local rural areas. They were transferred to the plastic trays containing tap water and cultured. The larvae were identified as *Culex* sp, *Anopheles* sp and *Aedes* sp by Acetocarmine staining method by an expert entomologist. The larvae were maintained at 27 °C ± 2 °C under photoperiod of 14:10 hours (Light/Dark) during the period of experiment. The laboratory maintained larvae were used for larvicidal activity. The stock solution was used to make different concentrations of the extracts ranging from 10 - 40%. Batches of twenty larvae were transferred into each disposable cup containing 100 ml of water. The mortality rate of larvae was studied by treating the larvae with 10, 20, 30 and 40 % extract at 27 °C ± 2 °C. Tap water is maintained as control. The larval mortality was observed till the 36th hour of exposure and percentage of mortality was calculated from the following formula:

Percentage mortality = No. of dead larvae/ No. of larvae introduced X 100

Statistical Analysis

Antibacterial activity evaluation was performed in duplicates and larvicidal activity was performed in triplicates. The means and standard deviations were calculated according to the standard methods for all the parameters. The P values were calculated by One-way ANOVA by SPSS Statistics Software Program. Data expressed in the tables are as mean ± S.D. The value of $P<0.05$ was considered to be statistically significant.

RESULTS

Protein Estimation

The protein concentration of each tissue extract was estimated. Muscle extract showed highest protein concentration of 340 µg/ml followed by skin extract with 240 µg/ml. Liver extract showed a protein concentration of 200 µg/ml, while gonads extract showed the least amount of protein with 94 µg/ml.

Antibacterial Activity

The zone of inhibition of five different bacterial strains against varied concentrations of the tissue extracts of *T. oblongus* was observed and measured (Table 1). The extracts showed maximum inhibition of the bacterial species at a concentration of 100 µl. Skin extract showed maximum zone of inhibition against *B. subtilis* and *K. pneumoniae* of 17 mm, whereas *P. aeruginosa* showed least inhibitory zone of 7 mm (Figure 1A). Muscle extract was ineffective against *B. subtilis* and *P. aeruginosa*, but showed maximum inhibitory zone against *S. aureus* of 11 mm followed by *E. coli* and *K. pneumoniae* of 10 mm, which is not a significant antibacterial activity (Figure 1B). Liver extract showed lesser antibacterial activity against *P. aeruginosa* and *B. subtilis*, but showed maximum zone of inhibition against *K. pneumoniae* of 22 mm followed by *E. coli* of 18 mm (Figure 1C). Gonads extract was

outstanding, showing the maximum antibacterial activity against most species except *B. subtilis*, which recorded the least zone of inhibition of 9 mm. Maximum inhibitory zone was recorded against *S. aureus* and *K. pneumoniae* of 28 mm followed by *E. coli* and *P. aeruginosa* of 24 mm (Figure 1D). Tetracycline, the positive control showed maximum inhibitory zone against *K. pneumoniae* of 30 mm, followed by *E. coli* with 23 mm and *S. aureus* with 22 mm. While *P. aeruginosa* and *B. subtilis* recorded a zone of inhibition of 20 mm against tetracycline.

Antifungal Activity

No zone of inhibition was formed by the tissue extracts against the fungal species and so they were reported to have no antifungal activity against the tested fungal pathogens.

Larvicidal Activity

The percentage of mortality of different larvae by various concentrations of the tissue extracts was calculated and tabulated (Table 2). Maximum death rate was observed at 40% concentration of all the extracts, while skin, liver and gonads extracts showed highest mortality at 30% too. Skin extract showed exceptional larvicidal activity in all concentrations, producing 100% death of all the larvae tested (Figure 6A). Muscle extract showed 100% mortality of all the larvae at 40% concentration, while producing a lesser range of larval mortality in *Anopheles* sp in different concentrations (Figure 6B). Liver and gonads extracts recorded the maximum i.e., 100% mortality at 30% and 40% concentrations respectively (Figure 6C & 6D). But still, under various concentrations, liver extract showed lesser mortality range in *Culex* sp, whereas gonads extract showed lesser range of mortality in *Anopheles* sp that is identical to muscle extract.

DISCUSSION

Marine environment, being a highly productive one, rich in various organisms which are explored for their ability to produce novel natural bioactive products that are of great use in treatment of various ailments. The profound ability to interact with their biological targets and novel mechanisms of action has validated an initiation towards drug discovery from natural products. Several drugs from marine organisms have entered the market as anti-cancer, anti-microbial, anti-tumour, anti-inflammatory, anti-oxidant, cytotoxic, anti-coagulant, immunomodulatory, antimetastatic, fibrinolytic agents and so on, after a series of clinical trials[27-31]. Discovery of antimicrobials from marine sources dates back to the 1950s while insecticidal agents from marine organisms developed in recent years is yet another milestone in the field of natural products' discovery[21,32-35]. Research on fishes for natural products' isolation has gained vital importance from several decades as fishes are known as main sources of proteins. Puffer fishes are reported to possess diverse bioactive potentials that holds considerable applications in many instances. The antimicrobial activities of different species of puffer fishes were earlier reported by Kumaravel *et al*., in 2011 and Khora *et al*., in 2013 and 2014[36-38]. Fouda *et al*., in 2005 reported the antitumour activity of the puffer fish *Arothron diadematus*[39]. Bragadeeswaran *et al*., in 2010 have reported the hemolytic properties of 6 species of puffer fishes from the south east Indian coast[40]. The antifouling activity of puffer fishes from Red Sea was reported by Soliman *et al*., in 2014[41]. In the present study, the antimicrobial and larvicidal activities of the puffer fish *T. oblongus* were evaluated and illustrated. The protein concentration of all the extracts was analyzed, in which the muscle extract showed maximum protein of 340 µg/ml while gonads extract had least protein of 94 µg/ml. The antibacterial activity of the tissue extracts of *T. oblongus* is quite encouraging, as the maximum zones of inhibition produced by gonads extract against the bacterial pathogens *S. auerus* and *K. pneumoniae* were 28 mm and 24 mm, which is quite closer to the effect of the antibiotic tetracycline whose maximum zone of inhibition was recorded to be 30 mm. The maximum inhibitory zone of 22 mm was recorded by liver extract which also accounts for a reasonable antibacterial activity. These results are contradictory to the study of Kumaravel *et al*., in 2011, which showed no noteworthy antibacterial activities, amidst showing very little activity by the liver extract. The skin extracts showed moderate antibacterial activity against few species in this study which is slightly in accordance with two separate studies of Khora *et al*., in 2013 and 2014, performed with 2 different puffer fish species, which showed convincing outcomes with the skin extract[37,38]. No antifungal activity by *T. oblongus* against the fungal pathogens is likely to the studies of Kumaravel *et al* in 2011, which also reported the absence of antifungal activity. But this is unlikely to the studies of Khora et al., in 2013 and 2014 which showed potential antifungal activity by the tissue extracts. Samidurai *et al*., in 2013 reported maximum larvicidal activities by the liver and gonads extracts and minimal activity by the skin extract which is quite contradictory to the present study that recorded maximum activity by the skin extract, followed by liver and gonads extracts. Hence, the study has vividly elucidated the biological potentials of the puffer fish *T. oblongus*, which could be applied for human welfare, if the bioactive compound could be purified by advanced techniques.

CONCLUSION

Concisely, the puffer fish *Takifugu oblongus* which harbors tetrodotoxin as a source of defense possesses remarkable bioactivities. There are possibilities for the toxin itself or its congeners to produce the above mentioned bioactivities, but this fact has to be confirmed with further studies. To summarize, the crude extracts of *T. oblongus* with natural chemical compounds, exhibited excellent biological properties which could be developed into novel potential drugs after purification and clinical trials.

ACKNOWLEDGEMENTS

The authors are grateful to the authorities of VIT University, Vellore, Tamil Nadu, India, for providing the required facilities, their constant encouragement and support.

REFERENCES

1. Sato A. The Search for New Drugs from Marine rganisms. *J Toxicol Toxin Rev*. 1996; 15(2): 171–198.
2. Grabley S, Thiericke R. Bioactive Agents from Natural Sources: Trends in Discovery and Application. In: Bhatia PK, Danielsson B, Gemeiner P, Grabley S, Lammers F, Mukhopadhyay A, et al., editors. *Thermal Biosensors, Bioactivity, Bioaffinitty*. Berlin Heidelberg: Springer; 1999: 101–154.
3. Tran TT, Munita JM, Arias CA. Mechanisms of drug resistance: daptomycin resistance. *Ann N Y Acad Sci*. 2015; 1354(1): 32–53.
4. Ahmed I, Sajed M, Sultan A, Murtaza I, Yousaf S, Maqsood B, *et al*. The erratic antibiotic susceptibility patterns of bacterial pathogens causing urinary tract infections. *Excli J*. 2015; 14: 916–925.
5. Kang HK, Seo CH, Park Y. Marine peptides and their anti-infective activities. *Mar Drugs.* 2015; 13(1): 618–654.
6. Schwartsmann G, Da Rocha AB, Mattei J, Lopes R. Marine-derived anticancer agents in clinical trials. *Expert Opin Investig Drugs*. 2003; 12(8): 1367–1383.
7. Simmons TL, Andrianasolo E, McPhail K, Flatt P, Gerwick WH. Marine natural products as anticancer drugs. *Mol Cancer Ther*. 2005; 4(2): 333–342.
8. Chun HG, Davies B, Hoth D. Didemnin B. The first marine compound entering clinical trials as an antineoplastic agent. *Invest New Drugs*. 1986; 4: 279–284.
9. Nonaka M, Satake H. Urochordate immunity. *Adv Exp Med Biol.* 2010; 708: 302–310.
10. Ellis A. Innate host defence mechanisms of fish against viruses and bacteria. *J Dev Comp Immunol.* 2001, 25(8): 827-839.
11. Cole AM, Weis P, Diamond G. Isolation and characterization of pleurocidin: An antimicrobial peptide in the skin secretions of winter flounder. *J Biol Chem*. 1997; 272(18): 12008-12013.
12. Fernandes JM, Smith VJ. A novel antimicrobial function for a ribosomal peptide from rainbow trout skin. J *Biochem Biophys Res Commun.* 2002; 296(1): 167-171.
13. Magnadottir B. Innate immunity of fish (overview). J *Fish Shellfish Immunol* 2006; 20(2): 137-151.
14. Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. *Food Research International*. 2011; 44: 523-529.
15. Zakon HH. Adaptive evolution of voltage-gated sodium channels: the first 800 million years. *Proc Natl Acad Sci U S A*. 2012; 109(1): 10619–10625.
16. Cusick KD, Sayler GS. An overview on the marine neurotoxin, saxitoxin: genetics, molecular targets, methods of detection and ecological functions. *Mar Drugs*. 2013; 11(4): 991–1018.
17. Hashiguchi Y, Lee JM, Shiraishi M, Komatsu S, Miki S, Shimasaki Y, *et al*. Characterization and evolutionary analysis of tributyltin-binding protein and puffer fish saxitoxin and tetrodotoxin-binding protein genes in toxic and nontoxic puffer fishes. *J Evol Biol*. 2015; 28(5): 1103–1118.
18. Nagashima Y, Mataki I, Toyoda M, Nakajima H, Tsumoto K, Shimakura K, *et al*. Change in tetrodotoxin content of the puffer fish *Takifugu rubripes* during seed production from fertilized eggs to juveniles. *Shokuhin Eiseigaku Zasshi J Food Hyg Soc Jpn*. 2010; 51(1): 48–51.
19. Howard Cohen. Puffer fish toxin for cancer pain, Atlantic breast cancer net (ABCN) newsletter, 2004 Sep15.
20. 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 in Med Chem.* 2003; 3: 785-787.
21. Samidurai K, Mathew N. Mosquito larvicidal and ovicidal activity of pufferfish extracts against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae). *Trop Biomed*. 2013; 30(1): 27–35.
22. Khora SS. Toxicity studies on pufferfish from tropical waters, *D. Ag. Thesis*. 1991; Tohoku University, Sendai, Japan: 129.
23. Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976; 72: 248–254.
24. Perez C, Pauli M, Bazerque P. An antibiotic assay by agar-well diffusion method. *Acta Biol Med Exp*. 1990; 15: 113-115.
25. Chenielle D, Lois R, Alison N, Sylvia M, John L, Mohammed A. Antibacterial and antifungal analysis of crude extracts from the leaves of *Callistemon viminalis*. *J of Med and Biol Sci*. 2009; 3(1): 1-7.
26. Subramaniam J, Kovendan K, Mahesh Kumar P, Murugan K, Walton W. Mosquito larvicidal activity of *Aloe vera* (Family:Liliaceae) leaf extract and *Bacillus sphaericus*, against Chikungunya vector, *Aedes aegypti*, *Saudi J of Biol Sci*. 2012; 19: 503–509.
27. Boopathy NS, Kathiresan K. Anticancer Drugs from Marine Flora: An Overview. *J of Oncol*. 2010; 2010: 1-18.
28. Montaser R, Luesch H. Marine natural products: a new wave of drugs? *Future Med Chem.* 2011; 3(12): 1475–1489.
29. Faulkner DJ. Marine natural products. *Nat. Prod. Rep.* 2001; 18: 1–49.
30. Hill RT. Marine natural products Biotechnology. *Biotechnol*. 9: 1-18.
31. Gudbjarnason S. Bioactive marine natural products. *Rit Fiskideildar*. 1999; 16: 107-110.
32. Berkholder PR, Burkholder LM. Antimicrobial activity of horny corals. *J. Science*, 1958; 127(3307): 1174-1175.
33. Nigrelli RS, Jakowsk S, Carlent I. Bioactive compounds from Sponges. In. *Zoologica*. New York: NY Publishing Company; 1959; 144-173.
34. Karthik L, Gaurav K, Rao KV, Rajakumar G, Rahuman AA. Larvicidal, repellent, and ovicidal activity of marine actinobacteria extracts against *Culex tritaeniorhynchus* and *Culex gelidus*. *Parasitol Res*. 2011; 108(6): 1447-1455.

35. Khanavi1 M, Toulabi PB, Abai MR, Sadati N, Hadjiakhoondi F, Hadjiakhoondi A, *et al*. Larvicidal activity of marine algae, *Sargassum swartzii* and *Chondria dasyphylla*, against malaria vector *Anopheles stephensi*. *J Vector Borne Dis.* 2011; 48: 241–244.
36. Kumaravel K, Ravichandran S, Sharmila Joseph FR, Manikodi D, Doimi M. *In vitro* Antimicrobial Activity of Tissue Extracts of Pufferfish *Arothron immaculatus* Against Clinical Pathogens. *Chin J of Nat Med.* 2011; 9(6): 446-449.
37. Mohana Priya K, Khora SS. Antimicrobial, Hemolytic and Cytotoxic activities of the Pufferfish *Arothron hispidus* from the Southeast Coast of India. *Int. J. Drug Dev. & Res*. 2013; 5(2): 317-322.
38. Sowmya J, Mohana Priya K, Niharika M, Khora SS. Bioactive Potential of Pufferfish *Arothron stellatus* collected from South East Coast of India. *Int. J. Drug Dev. & Res*. 2014; 6(3): 102-108.
39. Fouda FM. Anti-tumor activity of tetrodotoxin extracted from the Masked Pufferfish *Arothron diadematus*. *Egypt J of Biol*. 2005; 7: 1-13.
40. Mohan Raj M, Bragadeeswaran S, Suguna A. Studies on Haemolytic Properties of Pufferfishes from South East Coast of India. *Int Lett of Nat Sci.* 2014; 30: 11-18.
41. Soliman YA, Mohamed AS, Gomaa MN. Antifouling activity of crude extracts isolated from two Red Sea pufferfishes. *Egypt J of Aqua Res*. 2014; 40: 1–7.