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ANTIMICROBIAL, ANTICANCER PROPERTIES FROM VARIOUS TISSUES EXTRACTS OF PUFFER FISH AROTHRON STELLATUS FROM THOOTHUKUDI COAST

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ABSTRACT:

To evaluate the antimicrobial, anticancer activity of toxin present in the pufferfish *Arothron stellatus* collected from fish landing centre at Fishing harbour, Thoothukudi. Skin, intestine, muscle, and liver tissue extract of *A.stellatus* was extracted by using the 0.1% acetic acid. In vitro antimicrobial test was done by disc diffusion method against five bacterial and two fungal strains. To analyses the anticancer activity of tissues extract on the human lung cancer cell line A549. All the extract has shown inhibitory activity for antimicrobial test against bacterial and funcal strains, the liver extract has shown maximum zone against the S.aureus, E.coli, B.subtilis and K.pneumonia and the muscle extract has shown minimum against E.coli, P.aeruginosa and B.subtilis. The anticancer activity of the toxin increased lifespan by 34%, in addition to decreasing the number of cancer cells. Thus it can be concluded that bioactive compounds from this fish has excellent source for further development as a potential drugs.

Keywords: Puffer fish, ***A.stellatus,*** Antimicrobial activity, Anticancer activity.

INTRODUTION

Marine ecosystem covers nearly 70% of the earth's surface. Most of the marine organisms live in a hostile environment, having developed a well defence mechanism for their survival (Garson, 1989). Ocean offers a large biodiversity of fauna and flora which is estimated to be over 5,00,00 species or more than double of land species (Kamboj, 1999). India is among 12 mega biodiversity countries and 25 hotspots of the richest and highly endangered eco-regions of the world. Among the Asian countries, India is perhaps the only one that has a long record of inventories of coastal and marine biodiversity dating back to at least two centuries. However, these are so diverse in space, time and taxon that is almost responsible to review all records and reports (Venkataraman *et al.,* 2005). The puffer fishes are commonly known of all type of fish poisoning and has been recognized from ancient times. It is probably the most common fish intoxication along the coasts of Asia (Hwang *et al*., 2002). Puffer fish are generally believed to be the second most poisonous vertebrates in the world. The puffer fish takes its name from its tendency to puff its threat 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 blowfish, toadfish, swellfish, globe fish, bubble fish, balloon fish and sea squab (Torda *et al.,* 1973). The bacteria associated with some puffer fish produce powerful neurotoxin in their internal organs making them an unpleasant, possibly and lethal meal for any predator (Chun-Fai, 2004). Puffer will eat all type of food such as shrimp fish, clams, molluscs and crustaceans, etc. It is also important that they consume hard shelled crabs, mussels and shellfish in their diet to wear down their teeth and prevent them from overgrowing. Further more toxicity changes with age, sex, season and geographical variation (Homaira, 2010). Tetrodotoxin 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 (Seltzer, 1990 and Lau, 1995). During the World

War 2, the crude puffer fish extracts have been used for treating migraines and menstrual cramps. Cancer is one of the leading causes of human death in the world. Cell division is a physiological process that occurs in tissue. Balance between proliferation and programmed cell death is being under normal circumstances, usually in the form of apostosis by tightly regulating both processes. Certain mutations in DNA lead to cancer by disrupting the programmes that regulate the processes. Carcinogenesis is a process by which normal cells are transformed in to cancer cells. It is characterized by a progression of changes at both, cellular and genetic level, that reprogram a cell to undergo uncontrolled division, thus forming a malignant mass (tumor) that can spread to distant locations (Fearon,1990). Tumor growth is correlated with certain kinds of toxins. Tetrodotoxin is a naturally occurring potent toxin and highly selective sodium channel blocker (Fouda, 2005). The toxin name is subscribed to the puffer fish (tetrodontidae), the most commonly available source of Tatrodotoxin (Abd *et al.,* 2012). During the last decade, Tatrodotoxines has been a useful tool in identification, isolation and characterization of voltage-gated sodium chennels. Compared to morphine, tatrodotoxin was 3000 times more powerful with fewer side effects and no addictive qualities (Alonso *et al.,* 2003). Judicious selection of dietary fat has been suggested to prevent colon cancer (Elvevoll and James.,2000). It exhibits therapeutic potential including hepatoprotection and the inhibition of liver fibrosis and liver damage (Tieppo *et al.,* 2007). Hence the present study has been carried out to establish the occurrence of antibacterial, antifungal and anticancer activity and the possible chemical compounds from the methanol extract of muscle of *Arothron stellatus* collected from Thoothukudi Coast**.**

MATERIAL AND METHODS

SYSTEMATIC POSITION OF EXPERIMENTAL ANIMAL:

Kingdom : Animalia

Phylum : Chordata

Sub-phylum : Vertebrata

Class : Actinopterygii

Order : Tetraodontiformes

Family : Tetraodonitidae

Genus : *Arothron*

Species : *stellatus*

PLATE - 1

AROTHRON STELLATUS

COLLECTION OF SPECIMEN:

Specimens of puffer fish *Arothron stellatus* (Laecepede, 1958) were collected from fish landing centre at Fishing harbour, Thoothukudi. Then they were washed with seawater and transported to the laboratory in dry ice and stored in deep freezer at 20°C.

PREPARATION OF ACETIC ACID EXTRACT:

Specimens of *Arothron stellatus* was thawed and dissected out in to tissues like skin, intestine, muscle, and liver. Ten grams of each tissue was homogenized with 50ml of 0.1% acetic acid and were kept in water bath around 45°C for 10 minutes, cooled and centrifuged off. Then it was stored at the deep freezer for further used at -20°C (Kawabata, 1979).

TESTED ORGANISMS:

Bacterial species such as *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 737, *Escheridhia coli* MTCC 443, *Klebsiella pneumonia* MTCC 109,and *Pseudomonas aeruginosa* MTCC245 were maintained in Luria Bertani broth. Fungal species such as *Aspergillus niger* MTCC 1344 *Aspergillus flavus* MTCC 873.

Agar diffusion technique (Perez *et al.*, 1990):

The antibacterial activity of the acetic acid extracts of skin, intestine, muscle and liver of puffer fish *Arothron stelltus* were determined by the standard agar well diffusion assay Perez *et al.,* (1990), using Muller Hinton Agar. Petri plates were prepared by pouring approximately 20ml of Muller Hinton Agar medium and allowed to solidify. After solidification culture of each microbial pathogenic stain was swabbed to sterile cotton on surface of medium. Wells of 5mm diameter were punched using sterilized cork bores. The crude extracts were suspended in acetic acid at 2mg/10µl concentration for antimicrobial studies. Acetic acid extracts of skin, intestine, muscle and liver were tested with same aliquot 10µl of in each well and it was labelled. The plates were incubated for 24hrs at 37°C and solvent control was performed in each case. Areas of inhibited microbial growth were observed as clear zone around the well after 24hours. Antibacterial activity was measured as diameter of zone of inhibition excluding the well diameter.

ANTI FUNGAL ACTIVITY:

Agar diffusion technique (Bauer, 1966):

Antifungal activity of the crude acetic acid extract of marine puffer fish *Arothron stellatus* was determined by the standard method (Bauer, 1966) using potato dextose agar. Petriplates were prepared by pouring approximatly 20ml of potato dextose agar medium and allowed to solidify. After solidification culture of each fungal strain was swabbed with sterile cotton on the surface of medium.Wells of 5mm diameter were punched using sterilized cork bores. To each well 2mg/10µl of the samples of skin, intestine, muscle and liver were added and the solvent was used as the control. The petriplates was incubated at 30°C for 3 days. At the end of 72 hours, inhibition zone formed in the medium were measured in millimeters.

ANTI CANCER ACTIVITY:

Trypan Blue is a widely used assay (Burghardt *et al.*, 1994) for staining dead cells. A549 cancer cells were sub cultured in DMEM (Dulbecco's Modified Eagle's Medium) media supplement with 2 mM L-glutamine adjusted with 1.5 g/L sodium bicarbonate and 90% fetal calf serum incubated at 37 °C in 5% CO_2 incubator. 10µl of each sample like a puffer fish acetic acid extract such as skin, intestine, muscle, and liver was added to the A549 cancer cells separately added in PCR tubes & incubated at 37 °C for 24 hours. At the end of the treatment 20 µl of 10X tryphan blue was added. After 30 minutes, the viability was measured using haemocytometer.

$$\% \text{ of viability} = \frac{\text{Test OD}}{\text{Control OD}} \times 100$$

% of cytotoxicity = 100 - % of viability

RESULTS

Effect of acetic acid extract of *Arothron stellatus* on bacterial pathogens:

The skin extract showed antibacterial activity against that *Escherichia coli*, *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia and Pseudomonas aeruginosa*. The zone of inhibition ranging from 1mm to 5mm diameter at 2mg/10µl. In intestine, the zone of inhibition was measured as follows. The maximum activity was observed in *Klebsiella pneumonia* with 4.0mm diameter. *Escherichia coli* and *Bacillus subtilis* showed zone of inhibition 2mm diameter.Whereas *Pseudomonas aeruginosa* showed zone of inhibition 2.5mm diameter and 3mm diameter in *Staphylococcus aureus*. In muscle, zone of inhibition were observed as 3mm diameter in *Bacillus subtilis*, *Klebsiella pneumonia* and *Staphylococcus aureus*. Minimum acivity of the extract was seen in *Pseudomonas aeruginosa* as 1.5mm diameter. In liver, *Staphylococcus aureus* showed maximum zone of inhibition 8mm diameter and 7mm diameter in *Klebsiella pneumonia*. *Escherichia coli* and Basillus subtilis were sensitive with zone of inhibition 5mm diameter. Pesudomonas aeruginosa showed zone of inhibition 4.5mm diameter. (P< 0.05 – Nonsignificant), (Plate-2, Table-1, Fig-1).

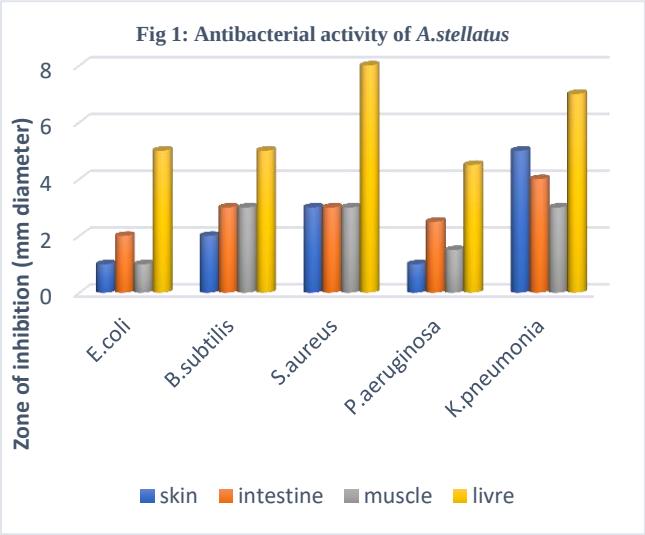
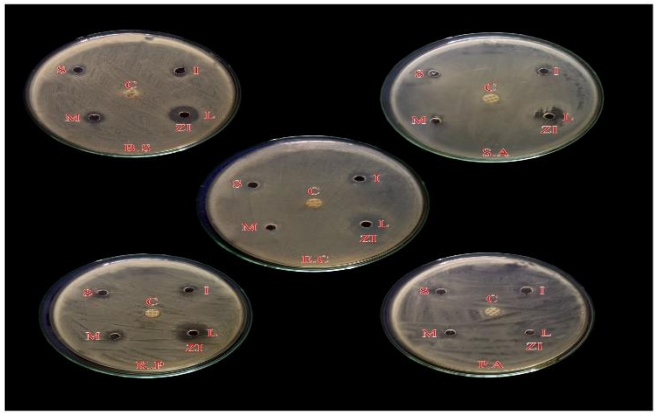


PLATE - 2

Muller Hinton Agar plates showing antibacterial activity of acetic acid extract of skin, intestine, muscle and liver of *Arothron stellatus* against bacterial strains



ZI - Zone of Inhibition
S - Skin
I - Intestine
M - Muscle
L - Liver

Table1:Inhibition zone of different tissues extract of *A.stellatus* against human pathogens(Mean±S.E).

	SKIN	INTESTINE	MUSCLE		LIVER
MEAN	2.4	2.9	2.3		5.9
S.D	1.59	0.76	0.97		1.35
S.E	0.71	0.34	0.43		0.60
	ONE WAY ANOVA				
SOURCE OF VARIATION	**DF**	**SS**	**MS**		**F**
BETWEEN	3	43.54	14.51		
WITH IN	16	51.15	3.20		
TOTAL	19	94.69	-		4.5
P		**NON-SIGNIFICANT**			

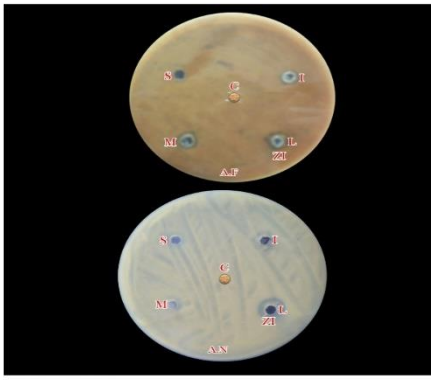
Effect of acetic acid extract of *Arothron stellatus* on fungal pathogens

Acetic acid extracts of skin, intestine, muscle and liver of puffer fish *Arothron stellatus* showed antifungal activity against *Aspergillus niger* and *Aspergillus flavus* at 2mg/10µl concentration. *Aspergillus niger* showed maximum zone of inhibition with 7.0mm diameter in liver extract. Skin and muscle extract inhibited *Aspergillus niger* with zone of inhibition 3.0mm diameter. Whereas intestinal extract exhibited antifungal activity with zone of inhibition 2.0mm diameter in *Aspergillus niger*.

In the case of *Aspergillus flavus* liver extract showed maximumm zone of inhibition with 7.5mm diameter. The muscle, intestine and skin extract showed zone of inhibition 3.5mm, 3mm and 2.0mm diameter respectively. Statistical analysis shows significant values (P< 0.05), (Plate-3 Table-2, Fig-2).

PLATE - 3

Potato dextrose ager plates showing anti-fungal activity of acetic acid extract of skin, intestine, muscle and liver of *Arothron stellatus* against fungal strains



ZI - Zone of Inhibition

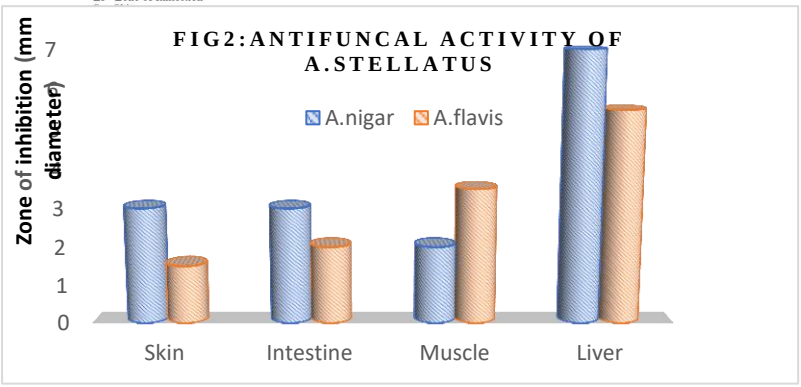
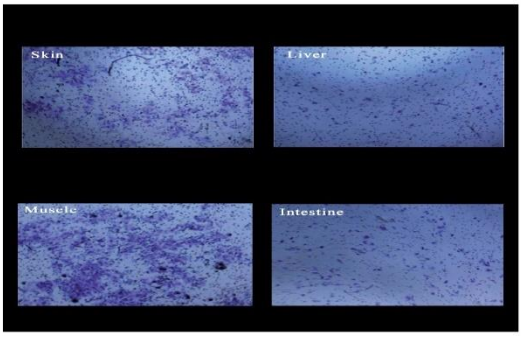


Table 2:

	SKIN	INTESTINE	MUSCLE	LIVER
MEAN	2.25	2.5	0.56	6.25
S.D	1.06	0.5	0.75	0.75
S.E	0.74	0.35	0.53	0.53
ONE WAY ANOVA				
SOURCE OF VARIATION	**DF**	**SS**	**MS**	**FS**
BETWEEN	3	21.33	7.11	1.09
WITH IN	4	28.64	7.16	
TOTAL	7	49.97	-	
P	**SIGNIFICANT**			

PLATE - 4

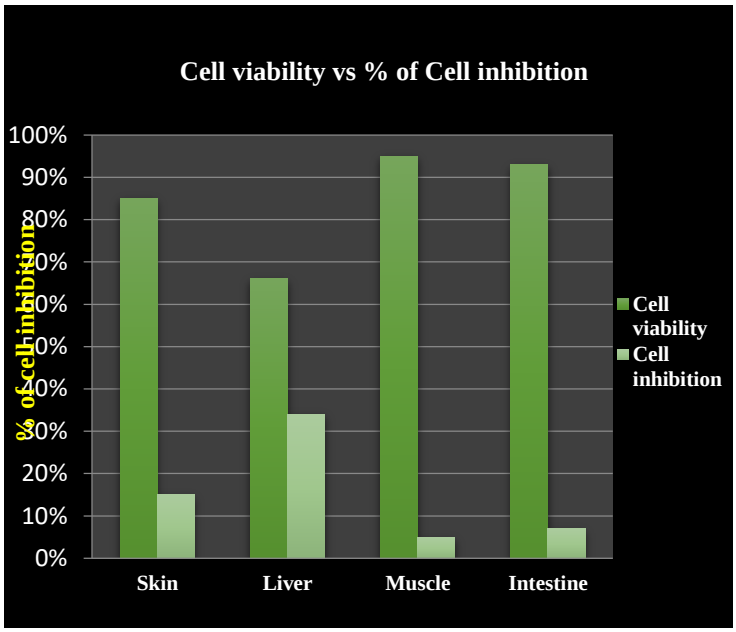
Photograph showing the effect of acetic acid extract of skin, intestine, muscle and liver of *Arothron stellatus* on A549 cell line



Anticancer activity of acetic acid extract of skin, intestine, muscle and liver of *Arothron stellatus*:

The percentage of cell vability was found to be 85%, 66%, 95% and 93% in skin, liver, muscle and intestine extract respectively at 10µl. The percentage of cell inhibition was noted as 15%, 34%, 5% and 7% in skin, liver, muscle and intestine respectively (Plate-4, Fig-4).

DISCUSSION



Marine organisms are yielding more variety with quantity and quality of bioactive compounds over the terrestrial organisms. Among different marine organisms which yield bioactive compounds, fishes also have a potentiality for the production of marine bioactive compounds. Antimicrbial compounds from marine fish *A.stellatus* inhibited the growth of five bacterial strains. Varying degrees of antimicrobial activity was found in crude extract of various tissues viz. skin, muscle, intestine and liver of puffer fish. Among the various extracts tested, acetic acid extract of liver exhibited strong.The diameter of inhibition of bacterial growth is regarded as an estimate of strength of the extract. The greatest zone of inhibition was shown by acetic acid extract of liver of *A.stellatus* of (7mm diameter) against *K.pneumonia*..Khora *et al*., (2013) found that the tissue extracts of *Arothron hispidus* shown antimicrobial activity against the bacterial and fungal strains. It showed maximum against of *E.coli* by skin extract and minimum was found against *P.vulgaris* in liver extract. The maximum antifungal activity was observed against *A.niger* and the minimum against *T.viridae*. Antibacterial activities of polyunsaturated fatty acid extracts from *Sardinella longiceps* and *Sardinella fimbriata*, extracts from both species showed inhibitory effect on the strains of *S.aureus, E.faecalis. P.vulgaris and S.enterica, S.fimbriata* (Chitra som and Radhakrishnan, 2011). Park *et al*., (1998) reported that endogenous peptide with antimicrobial activity from fish was found mainly from the skin and its secretions. He also found that the antimicrobial peptide derived from Histone H_2A in catfish *Parasilurus asotus*. The antimicrobial property of mucus against the various pathogens has been demonstrated in rock fish *Sebastes schdegelii.* The crude and frachais of venom gland and gonad scorpian fish *Scorpaenopsis venosa* were potent against *V.harveyi* (Sasikala *et al*., 2013). An antimicrobial validatory screening of puffer fish is done in such a way, *Arothron immaculatus* a puffer fish skin and liver extract were subjected for antimicrobial assay. The results confirmed a positive test against most of the pathogens used. Maximum antimicrobial effect against *Staphyloccocus aureus* of 2.5mm in liver extract and 9.8mm of antibacterial effect against *Vibrio cholera* in skin extracts is reported. So this experiment confirms that puffer fish is a source of antimicrobial potence (Kumaravel *et al*., 2011).The acetic acid extract was subjected to antifungal activity on two fungal strains such as *Aspergillus niger* and *Aspergillus flavis*. In skin and muscle *Apergillus niger* showed the minimum zone of inhibition of 3mm diameter. Intestine extract exhibited antifungal activity with zone of inhibition 2mm diameter in *A.niger*. The liver extract showed maximum zone of inhibition of 7mm and 7.5mm diameter in *Aspergillus niger* and *Aspergillus flavis*. Similar result was shown by Samanta *et al*., (2013). The extract of the fish *A.hispidus* produced strong activity against *A.niger* in the skin extract and the minimum was observed against *T.rubrum* in the liver extract with zone of inhibition 11.1mm. It indicated that the fish extract possess highly polar based bioactive compound. Reports revealed that antimicrobial activity of skin acetic acid extract of *A.stellatus* has shown activity against various bacterial and fungal organisms. The antibacterial activity was found maximum against *E.coli* and *S.aureus* and minimum against *K. pneumoniae,* antifungal activity was observed maximum against *A.nigar* and *A.flavus* and minimum activity against *T.viridae* (Soumya *et al.,* 2014). In this study the acetic acid extract of *A. stellatus* showed potential effect on the tested fungal strain. There is a correlation that exists between the antifungal and antibacterial activity of the test extract. The variations in the extracts that occurred may be due to physical parameters of the environment and the chemical composition in the fish. In the present investigation, among the various tissue extract tested on A549 cell line, liver extract exhibited strong anticancer activity. The percentage of cell viability from the result clearly showed that acetic acid extract of liver has cytotoxic effect on A549 cell line. Reports have suggested that anticancer of extracts of jellyfish *Chrysaora quiniquecirrha*, that the extracted peptides could be responsible for the anticancer potential on human lung cancer cell lines (A549). It study is focuses on the anticancer potential of marine

species with certain pharmaceutical interest, which could confer anticancer activity (Suganthi *et al*., 2014). Nielson, (2004) determined that the ability of EAC cells in resisting drugs depends on the ability of the cell membrane to prevent the drug from penetrating the cell in addition to controlling the surrounding media. He also demonstrated the ability of P-glycoproteins found in the drug, reducing the chance of drug penetration. Here the binding of TTX to P-glycoproteins guarding the Na^+ chennal may have reduced the influx of Na^+ to the cell, which in return could have reduced its invasiveness.

CONCLUSION

There is no doubt that the sea possesses plenty of metabolites and constitutes a potential source of new drugs for fighting antibiotic-resistant infection and other deadly diseases. In fact, great effects of scientists at different parts of the world have become extracted various kinds of drug for several diseases in recent years. Therefore every discovery of metabolites is considered important because it adds to the knowledge base of compounds unique to the marine environment. Currently, the number of natural products is increasing, however very few compounds have reached the market. Since biodiversity of the marine environment for exceeds that the terrestrial environment, research on the use of marine natural products as pharmaceutical agents has been steadily increasing. The percent study revealed that acetic acid extract of skin, liver, intestine and muscle of puffer fish *Arothron stellatus* had obvious antimicrobial, antifungal, and anti-cancer activity. Thus it can be concluded that biotoxins from the *A.stellatus* has an excellent source for further development as a potential drugs.

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