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Determination of Antibacterial Activity of Marine Puffer Fish *Arothron Immaculatus* Collected From Thoothukudi Coast

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Abstract

The antibacterial activity of crude acetic acid extracts of puffer fish *Arothron immaculatus* against ten bacterial strains has been evaluated. Skin, liver, muscle, intestine and ovary extracts of *Arothron immaculatus* prepared by using acetic acid. The antibacterial test was performed by Agar diffusion technique. The ovary extract showed maximum activity with zone of inhibition with 14.0 mm radius at 10mg/10µl in *Shigella flexneri* and the muscle extract showed minimum zone of inhibition with 6.0 mm radius at 10mg /10µl in *Pseudomonas sp.* The antibacterial activity of tissue extracts of *Arothron immaculatus* was compared with the standard antibiotics like Malachite green, Penicillin and Streptomycin. The results indicated that the Skin, liver, muscle, intestine and ovary tissue extracts of *Arothron immaculatus* may have potent antibacterial compounds that can be further explored and utilized for the welfare of mankind.

Key words : *Arothron immaculatus*, antibacterial activity, *Bacillus cereus* and Zone of inhibition.

INTRODUCTION

Ocean offers a large biodiversity of fauna and flora which is estimated to be over 5,00,000 species and more than double that of the land (Anand *et al.*, 1997). This rich diversity of marine organisms assumes a great opportunity for the discovery of new bioactive substances. Thus the marine environment is an exceptional reservoir for bioactive natural products, many of which exhibit structural features that are not found in terrestrial natural products (Johansson and Soderhall, 1985).

Marine organisms are a rich source of structurally novel and biologically active metabolites. Primary and secondary metabolites produced and stored by these organisms may be potential bioactive compounds of interest in the pharmaceutical industries. The number of natural products isolated from marine organisms increases rapidly (Faulkner, 2002 and Proksch *et al.*, 2006). Many classes of natural products from marine sources exhibiting antitumour, anti-leukaemia, antibacterial and antiviral activities have been reported worldwide (Khora, 2013).

Fishes are one of the diverse source of natural products and bioactive compounds with over 40,000 known species. They combat infections caused by viruses, bacteria, fungi and parasites that are similar to those of humans and other vertebrates. Many species of marine fish have been reported as ithyocrinotoxic (Halstead, 1978), releasing into the water toxic secretions.

Tetraodontidae is diverse with species such as Puffer fish, Balloon fish, Blowfish, Bubble fish, Globe fish, Swell fish, Toad fish, toadies, Honey Toads and Squab (Mills and Passmore, 1988; Ramaiyan and Senthil kumar, 1998; Froese and Pauly, 2007). They are commonly distributed in the tropics, but are relatively uncommon in temperate regions and completely absent from cold water. There are 189 species of puffer fishes and 28 genera in the family Tetraodontidae (Oliveira *et al*., 2006). Puffer fishes are the second most poisonous vertebrate in the word, the first being a "Golden Poison Frog" (Keiichi *et al*., 1998).

Puffer fish is known to carry tetrodotoxin (TTX) (Bilecenoglu *et al*., 2006; Kasapidis *et al*., 2007 ; Sabrah *et al*., 2006) which is known a non-protein organic compound (amino perhydroquinazoline) and one of the strongest marine paralytic toxins today. The toxin has only occasionally been detected in the muscles of these fishes. The toxin is produced by several bacteria species including *Mycobacterium arabino galatanolyticum*, *Serratia mascescens*, *Vibrio alginolyticus* and *Bacillus sp*. (Yu *et al*., 2001 and Wu *et al*., 2005).

The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from natural source. Many antimicrobial peptides shows a high specificity for prokaryotes and a low toxicity for eukaryotic cells and their mode of action is considered unlikely to lead to the development of resistance. These properties have favoured their investigation as potential new antibiotics (Bax *et al*., 2000).

Anionic antimicrobial peptides/ proteins (AAMPs) were first reported in the early 1980s. Antimicrobial peptides (AMPs) have become recognized important components of the nonspecific host defense or innate immune system in a variety of organisms including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals (Anitha and Sharath, 2011). Anbuchezhian *et al.,* (2011) analyzed that antimicrobial peptide from the epidermal mucus of estuarine cat fishes. Antibacterial activity in tissue extracts has been demonsrated in several fish species (Austin and Mclntosh, 1988).

Knouft *et al*., (2003) has reported endogenous peptides with antimicrobial activity from fish mainly from the skin and its secretions. The antibacterial activities of fatty acids in general have been noted in several pioneering studies (Kabara *et al.,* 1972; Willet and Morse, 1966; Miller *et al.,* 1977; Sun *et al.,* 2003).

In India, studies on the antibacterial activity of puffer fish are very limited and it remains unexploited. The aim of the present study is to evaluate the antibacterial activity of skin, liver, muscle, intestine and ovary of *Arothron immaculatus* collected from Thoothukudi coast.

MATERIALS AND METHODS

Collection of Specimen:

Specimens of the puffer fish *Arothron immaculatus* were collected from fish landing centre at fishing harbour, Thoothukudi. They were kept in ice-box and transported to the laboratory and maintained in a deep freezer at -20ºC until use.

ANTIBACTERIAL ACTIVITY

Preparation of Acetic acid extract:

Specimen of *Arothron immaculatus* was thawed and dissected out into tissues like skin, liver, muscle, intestine and ovary. Ten grams of each tissue was homogenised with 50ml of 0.1% acetic acid and were kept in water bath around 45ºC for 10 minutes, cooled and centrifuged off. Then it was stored in the deep freezer at -20ºC for further use.

BACTERIAL STRAINS

The reference strains used to test antimicrobial activity includes *Bacillus cereus(BC), Vibrio cholerae (01 (VC01)), Vibrio cholerae(0139)(VC0139), Escherichia coli(EC), Pseudomonas aeruginosa(PA), Aeromonas hydrophila(AH), Salmonella typhi(ST), Shigella flexneri(SF) ,Pseudomonas sp(P.sp)* and *Staphylococcus aureus (SA).*

AGAR DIFFUSION TECHNIQUE (Acar, 1980)

1.0 ml of 12 hr old nutrient broth was transferred into the sterilized petridishes .Petri plates were prepared by pouring approximately 20 ml of Muller Hinton Agar Medium and allowed to solidify. Sterilized paper discs prepared from Whatmann No 1 were used for loading acetic acid extract. The paper discs were loaded with different concentrations viz 10mg/10µl, 1mg/10µl and 0.1mg/10µl. The plates were incubated for 24hr at 37ºC and solvent control was performed in each case. Areas of inhibited microbial growth were observed as clear zone around the paper disc after 24 hours.

DETERMINATION OF ACTIVITY INDEX:

Activity index was calculated by following the method of Singh *et al*., (2002)

$$\text{Activity index (A. I)} = \frac{\text{Mean of zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

ANTIBIOTIC SENSITIVITY ASSAY

To test the sensitivity of bacterial strains to standard antibiotics, agar diffusion technique was followed by using commercial antibiotics. The antibiotics such as Malachite green, Penicillin and Streptomycin (10mg/10µl) were loaded on filter paper discs. The zone of inhibition was measured after 24 hours.

RESULTS

The crude tissue extracts (Skin, Liver, Muscle, Intestine and Ovary) of *Arothron immaculatus* were screened against ten human pathogenic bacteria for testing their antibacterial activities. The maximum zone was observed against the *Salmonella typhi* in the

ovary extract of *Arothron immaculatus* and minimum zone was observed against *Shigella flexneri* in the muscle extract. The results were shown in Table 1 and plates 1 – 5. The inhibition zones of the extracts were compared with standard antibiotics Malachite green, Penicillin and Streptomycin (Plate 6). Activity index of the tissue extracts varies from maximum 0.6 for ovary extract (*Salmonella typhi*) to minimum 0.27 for muscle extract (*Shigella flexneri*) (Figure 1- 5).

Table – 1

Activity of acetic acid extracts of skin, liver, muscle, intestine and ovary of *Arothron immaculatus* against bacterial strains

Bacterial Strains	Control	10 mg/ 10µl.					1 mg/ 10µl.					0.1 mg/ 10µl.				
		Skin	Liver	Muscle	Intestine	Ovary	Skin	Liver	Muscle	Intestine	Ovary	Skin	Liver	Muscle	Intestine	Ovary
BC	-	++	+++	+++	+++	+++	+	+++	++	++	++	+	++	++	++	+
VC(01)	-	++	+++	++	+++	+++	+	++	++	++	++	+	++	+	+	++
VC(0139)	-	++	+++	+++	++	++	+	++	++	++	++	+	+	+	+	+
EC	-	+	+++	++	++	+++	+	+++	++	+++	++	+	++	+	++	+
PA	-	+	+++	++	+++	++	+	++	++	++	++	+	++	++	++	+
AH	-	+	+++	++	+++	++	+	+++	++	++	++	+	++	+	+	+
ST	-	+	+++	+++	++	+++	+	+++	++	++	+++	+	++	++	+	++
SF	-	+	+++	+++	+++	++	+	++	++	++	++	+	+	++	+	++
P.sp	-	+	++++	++	+++	++	+	+++	++	++	++	+	++	+	++	+
SA	-	+	+++	+++	++	+++	+	++	++	+	++	+	++	++	+s	++

Zone of inhibition mm (radius), + 5– 8, ++ 8 – 11, +++ 11 – 14

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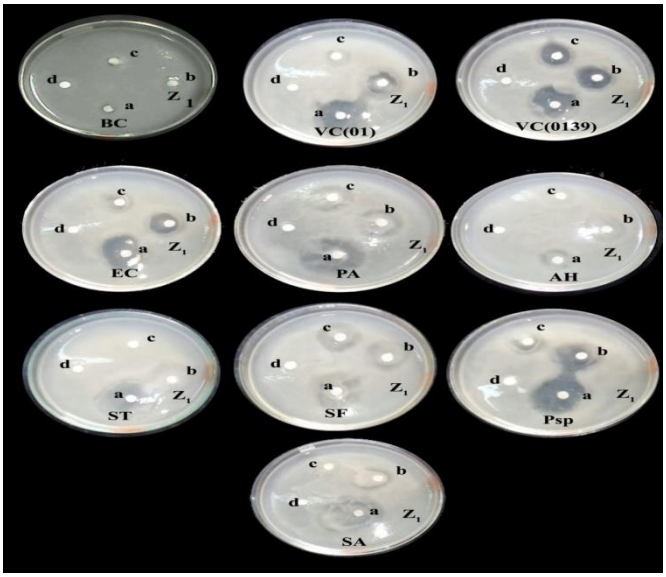

Plate 1 : Muller Hinton Agar plates showing antibacterial activity of acetic acid extract of skin of *Arothron immaculatus* against bacterial strains

Z_1- zone of inhibition
a – 10mgl / 10µl
b – 1 mg / 10µl
c - 0.1 mg / 10µl
d - control

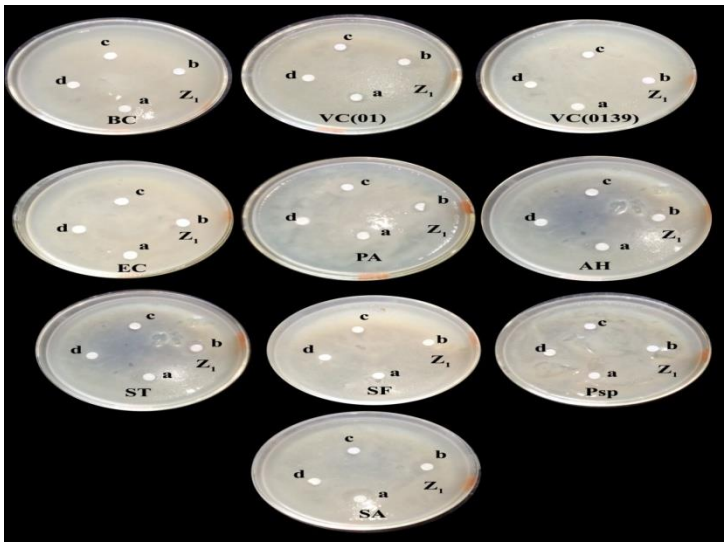

Plate 2 : Muller Hinton Agar plates showing antibacterial activity of acetic acid extract of liver of *Arothron immaculatus* against bacterial strains

Z_1- zone of inhibition
a – 10mgl / 10µl
b – 1 mg / 10µl
c - 0.1 mg / 10µl
d - control

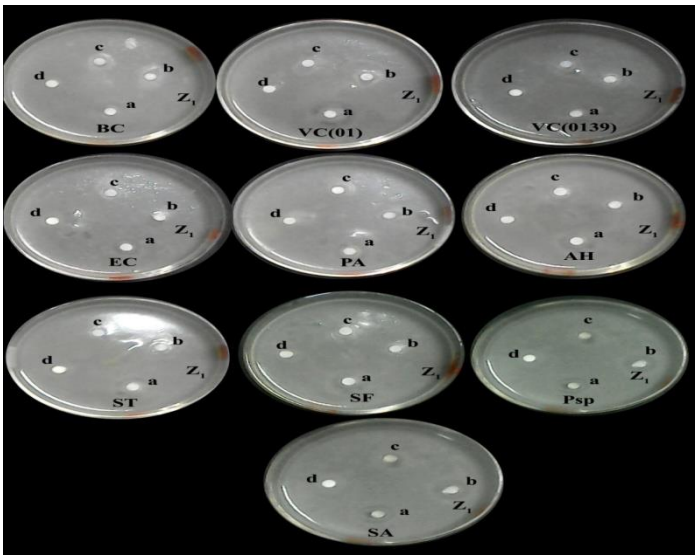

Plate 3 : Muller Hinton Agar plates showing antibacterial activity of acetic acid extract of muscle of *Arothron immaculatus* against bacterial strains

Z_1- zone of inhibition
a – 10mgl / 10µl
b – 1 mg / 10µl
c - 0.1 mg / 10µl
d - control

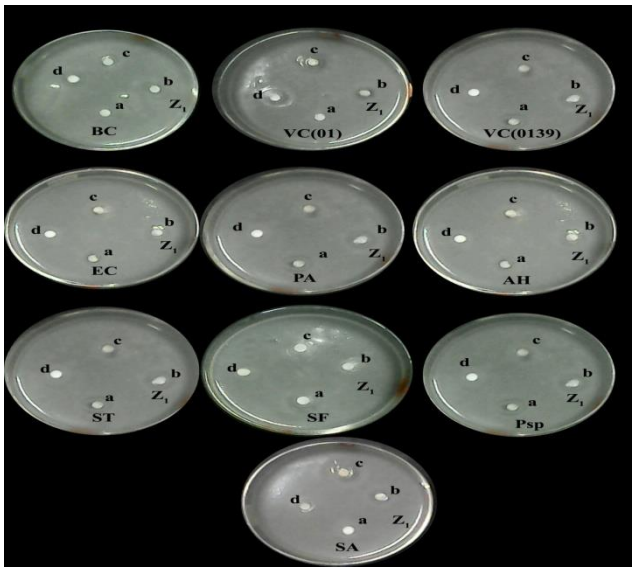

Plate 4 : Muller Hinton Agar plates showing antibacterial activity of acetic acid extract of intestine of *Arothron immaculatus* against bacterial strains

Z_1- zone of inhibition
a – 10mgl / 10µl
b – 1 mg / 10µl

c - 0.1 mg / 10µl
d - control

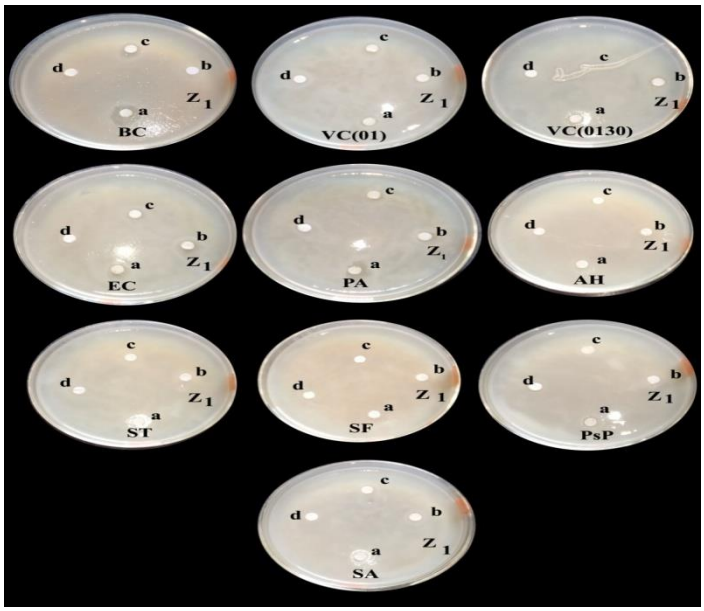

Plate 5 : Muller Hinton Agar plates showing antibacterial activity of acetic acid extract of ovary of *Arothron immaculatus* against bacterial strains

Z_1- zone of inhibition
a – 10mgl / 10µl
b – 1 mg / 10µl
c - 0.1 mg / 10µl
d - control

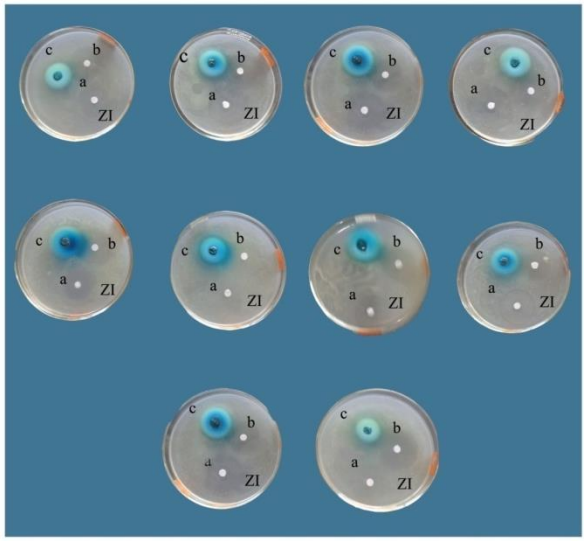

Plate 6 : Muller Hinton Agar plates showing antibacterial activity of commercial antibiotics penicillin, streptomycin, malachite green on bacterial strains

ZI - Zone of inhibition
a - penicillin
b - streptomycin
c - malachite green

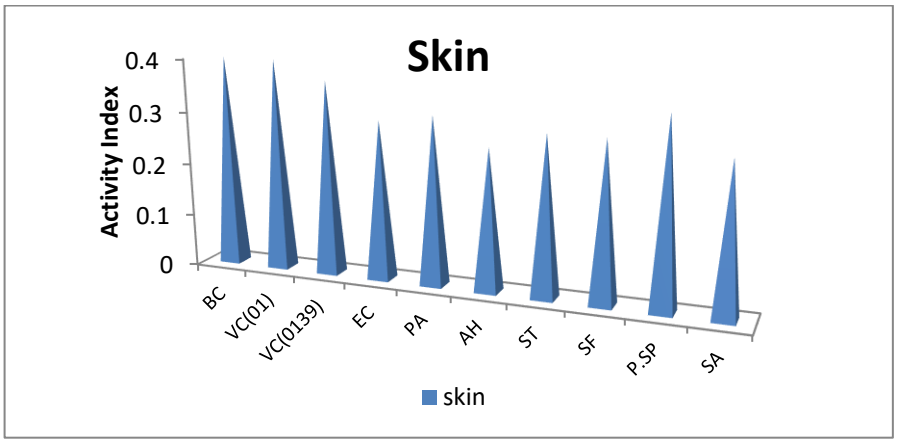

Figure 1 : Activity index of crude acetic acid extract of skin of *Arothron immaculatus* against bacterial strains

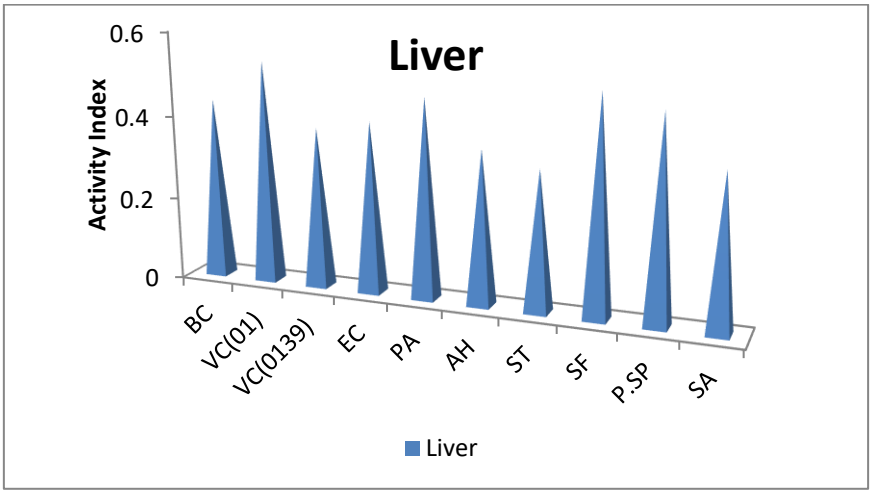

Figure 2 : Activity index of crude acetic acid extract of liver of *Arothron immaculatus* against bacterial strains

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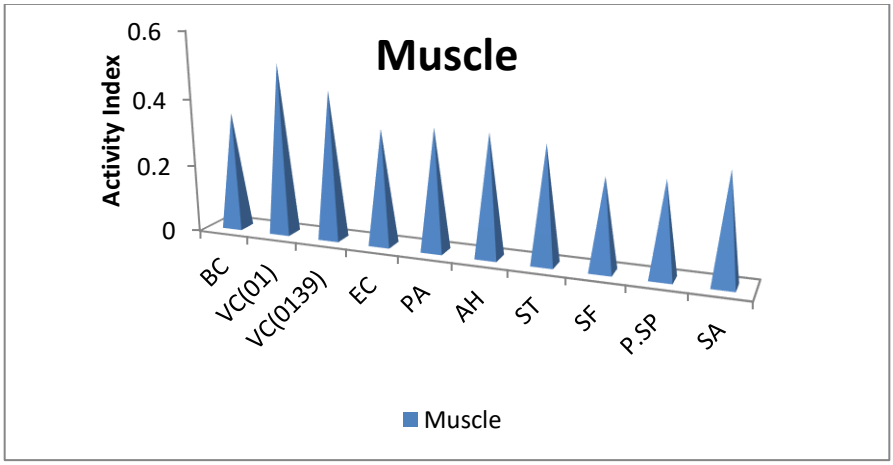

Figure 3 : Activity index of crude acetic acid extract of muscle of *Arothron immaculatus* against bacterial strains

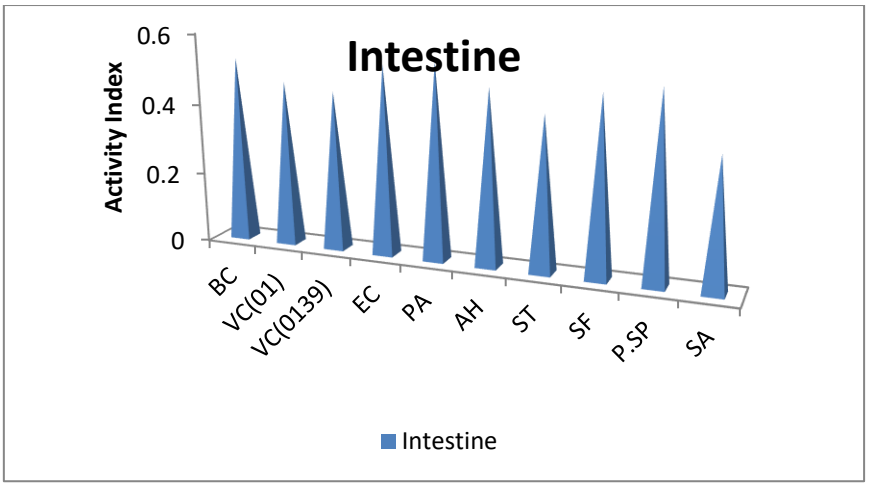

Figure 4 : Activity index of crude acetic acid extract of intestine of *Arothron immaculatus* against bacterial strains

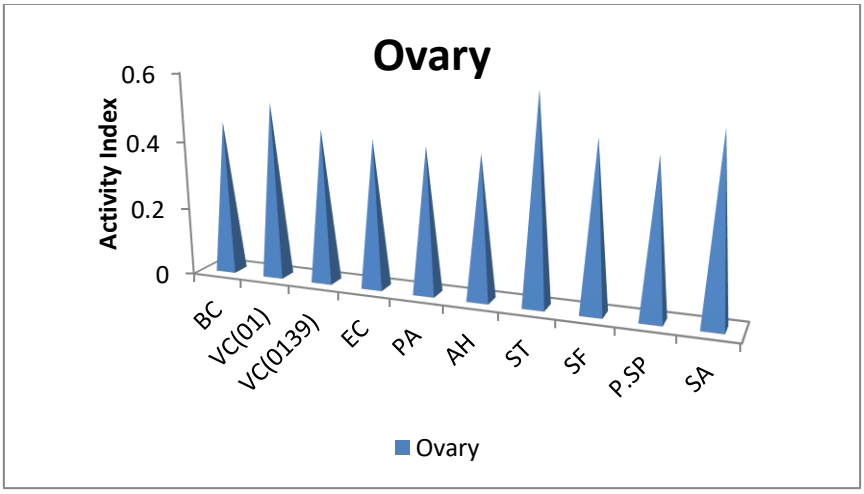

Figure 5 : Activity index of crude acetic acid extract of liver of *Arothron immaculatus* against bacterial strains

DISCUSSION

Most of the marine creatures have been found to exhibit antimicrobial activity against human bacterial pathogens as well as fungi and viruses and most of the antibacterial agents have derived from marine sources (Mohan Raj *et al.*, 2014). 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 (Hencock and Scott 2000).

The mode of action of antimicrobial agents also play a vital role in the bacterial susceptibility. This is because different antimicrobial agent affects the microorganisms differently. Most of reported antimicrobial peptides typically have strong antimicrobial activity against a wide range of Gram – positive bacteria but very weak or no activity against Gram – negative bacteria (Mitta *et al*., 2000). The antimicrobial property of mucus against the various pathogens has been demonstrated previously in rock fish(Kitani *et al*., 2008).

Bacteriostatic compounds from marine puffer fish *A. immaculatus* collected from Thoothukudi coast inhibited the growth of ten bacteria viz *B. cereus, V. cholerae* (01), *V. cholerae* (0139), *E. coli, P.aeruginosa, A. hydrophila, S.typhi, S.flexneri, Pseudomonas sp.* and *S. aureus*. Varying degrees of antibacterial activity were found in crude acetic acid extracts of skin, liver, muscle, intestine and ovary. Acetic acid extract of ovary exhibited strong antibacterial activity against bacterial strains.

The diameter of inhibition of bacterial growth is regarded as an estimate of strength of extracts. In the present study the tested bacterial strains showed different patterns of inhibition zone. The maximum zone was observed against the *Salmonella typhi* in the ovary extract of *Arothron immaculatus* and minimum zone was observed against *Shigella*

flexneri in the muscle extract. The findings of Kumaravel *et al*., (2011); Mohana Priya *et al*., (2013); Khora *et al*., (2013); Selvi and Joslin (2016) lended support to our results.

Kumaravel *et al*., (2011) reported that liver and skin extracts of *A. immaculatus* showed antibacterial activity against *S. aureus* and *V. cholerae*. Mohana Priya *et al*., (2013) reported that maximum zones against *E. coli*, in the skin extracts of *A. hispidus* and the minimum zone was observed against *Proteus vulgaris* in the liver extract. Khora *et al*., (2013) reported that the crude acetic acid extract of skin of puffer fish *A. stellatus* exhibited maximum inhibitory activity against *E. coli* and *S. aureus* and minimum activity against *K. pneumoniae*. Selvi and Joslin (2016) reported that the acetic acid extracts of liver and muscle of puffer fish *Chelonodon patoca* showed maximum activity (12. 0 mm) in *Pseudomonas sp*. and in *Shigella flexneri* (11.0 mm) respectively. The results clearly indicate that the toxins present in the fish having bioactive compounds that may be used for therapeutic needs.

CONCLUSION

In the present study, the skin, liver, muscle, intestine and ovary extracts of puffer fish *Arotrhron immaculatus* have been examined for their bioactivity. These results revealed that the tissue extracts has some valuable bioactive compounds. So this study paves the way for further investigation on the pharmacological composition of puffer fish to discover potential chemotherapeutic agents.

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