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Research Article

An *in vitro* antimicrobial activity and bioactivities of protein Isolated from Rabbit fish - *Siganus javus*.

N. Prithiviraj* and D. Annadurai

Annamalai University, Faculty of Marine Sciences, Centre of Advanced Study in Marine Biology, Parangipettai - 608 502, Tamilnadu, India

*Corresponding author

Abstract

Objective: To explore antimicrobial activity and bioactivities of protein from Rabbit fish *Siganus javus.* **Methods:** A live *S. javus,* sp collected from parangipettai coastal waters, Tamilnadu, India and the dorsal and anal spine were cut approximately 3 - 5 mm from the base and homogenized in normal saline at pH 7.4. The Partial purification of protein was carried out using DEAE cellulose. Antimicrobial properties of the rabbit fishes were tested against 5 pathogenic bacteria and 5 pathogenic fungi. The hemolytic study of rabbit fish venom was primarily done by chick, goat, sheep and human blood erythrocytes was recorded. Further characterization of crude extract was done through FT - IR. The amino acid content through HPLC analysis was also studied. Anticancer activity was done by Hep_2 and Vero cell lines **Results:** The results of the present investigation showed that *S. javus* rabbit fish having remarkable antimicrobial activity. The spine crude extracts showed a very strong hemolytic activity in human 16 HU and sheep blood 14 HU, moderate activity in chick blood 10 HU and lowest activity in goat blood 8 HU respectively, FTIR analyze revealed the presence of bioactive compounds signals at different ranges. And its amino acid composition was rich in serine and glycine, but low in basic amino acids. The IC_{50} on Hep_2cells was found to be 51 µg/ml where on Vero cells 50 µg/ml a respectively. The control group doesn't show any considerable cell death. **Conclusions**: Rabbit fish may also contain some biologically active agents which have hemolytic, antimicrobial and anti - cancer activity. Further studies will fulfill for purification and structural elucidation.

Keywords: *Siganus javus*, Antimicrobial properties, Hemolytic assay, FT-IR, HPLC.

Introduction

Siganus javus, siganidae family are popularly called as rabbit fish, fox face or spine foot. Siganids are much appreciated food items by people in the Indo-pacific and eastern mediterranean (Hara *et al*., 1986; Lam, 1974). Works on siganids from Indian waters are scanty, being restricted to that of traditional trap fishery (Mohan and Lal, 1985), food and feeding (Balasubramanyam and Natarajan, 1980). There are 30 species recorded the world over, and are distributed in reefs among sea grasses, mangroves, and estuaries and also in shallow lagoons of tropical and subtropical coastal environments including southern Korea, Japan, Southeast Asia, Australia, Indo - Pacific from the Arabian Gulf to the Indo - Malay region, Hong Kong, Taiwan and Red Sea (Bariche, 2005; Lam, 1974; Randall *et al*., 1990; Woodland, 1983; Woodland, 1990). They feed on filamentous algae

and sea grasses. Twenty eight nominal species are currently recognized in that family based on morphology and colour patterns (Randall and Kulbicki, 2005). Fishes of the family siganidae exhibit uniformity in those characters (*i.e*. numbers of fin spines and rays, tooth shape, tooth count) spines (13 dorsal, seven anal, 2 pectoral) are venomous, which the systematic of fishes usually rely on. *S. canaliculatus, S. javus, S. lineatus, S. stellatus* and S. *vermiculatus* are the common species found in India (Jaikumar, 2012). Two species *S. canaliculatus* (white - spotted spine foot) and *S. javus* (streaked spine foot) are found in the GOM region (Muralitharan, 1999). The majority of fishes of the family have bright and unique colour patterns have been exploited for defining species boundaries, but higher - level classification essentially relies on gross body proportions, shape of tail, and length of snout (Woodland, 1990). Siganids are economically important fishes (Woodland, 1983) and attracted the attention of mariculturists of the Indo - Pacific regions mainly because of their herbivorous food habits, rapid growth and commercial value (Lam, 1974; Randall *et al*., 1990).

Some of these venomous fish awoke special interest, since they represent more than 50 % of the venomous vertebrates which are often involved in human accidents. Envenomation occurs when the victim treads on or Handles the fish and has the skin perforated by the spine, which releases the venom into the wound Injuries provoked by *S. javus* family fishes are characterized by immediate, local, intense pain, soft tissue edema and a variable extent of bleeding However there is very little information on the rabbit fishes venom, Hence the present study was carried out on antimicrobial activity and bioactivities of protein from rabbit fish *Siganus javus.*

Materials and Methods

Collection of fish sample

The live species of *S. javus* were collected from Parangipettai- Annakovil landing center South east coast of India and were immediately taken to laboratory figure1.

Preparation of the Crude Extract

Fishes were chilled at - 20°C for 10 - 20 minutes and then decapitated; the dorsal and anal spine were cut approximately 3 - 5mm from the base and homogenized in normal saline at pH 7.4 figure 2. The supernatant was centrifuged at 6000 x g for 15 minutes at 4°C to remove insoluble material. The pellet was discarded and the supernatant was collected and lyophilized.The crude extract (spine venom) was stored at - 20°C for further analysis. All the steps were carried out in cold room at 4°C.

Partial purification of crude protein: Partial purification of the crude extract *S. javus* was carried out using DEAE Cellulose Anion Exchange chromatography according to the procedure (Stempein *et al*., 1970).

Estimation of Protein

Amount of protein in the sample was estimated according to the method (Lowry *et al*., 1951) with BSA as a Standard.

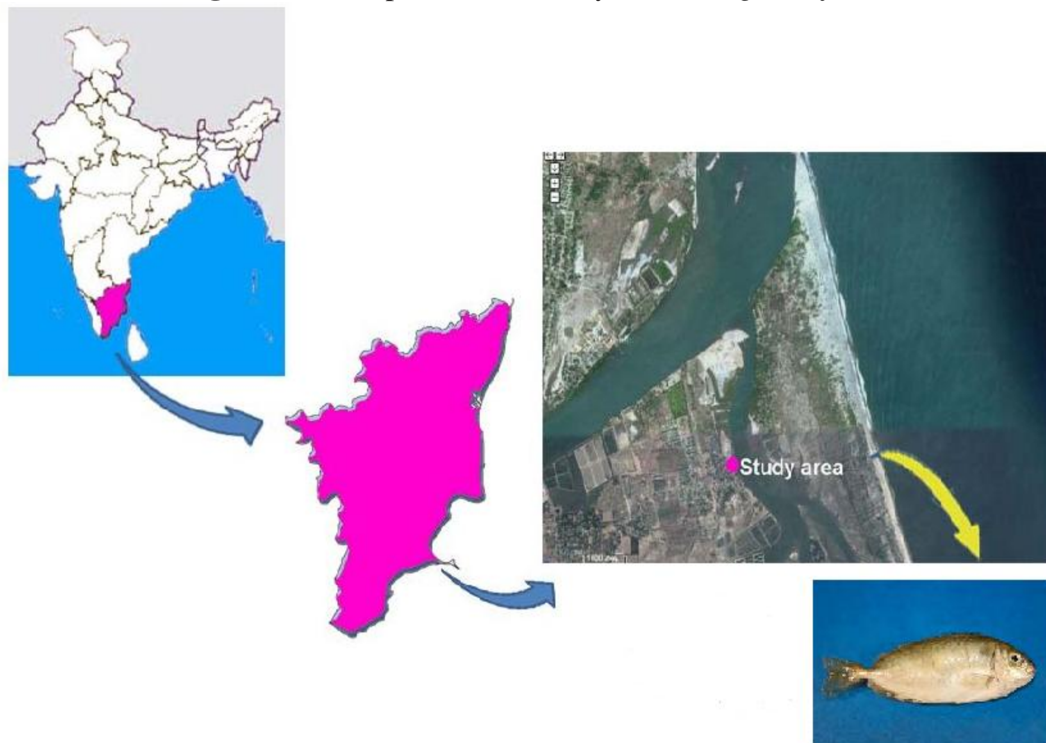
Microbial Strains Used

Antibacterial effect of was determined against different bacterial strains viz. *Pseudomonas* sp, *Streptococcus aureus, Vibrio cholerae, Bacillus* sp, *E. coli* and *Lactobacillus brevis* similarly Antifungal effect was determined against 5 different fungal strains viz. *A. flavus, A. niger, Candida albicans A. oryzae and A. sojae.* These pathogenic strains were obtained from the Department of Medical Microbiology (*Raja Muthiah Medical College hospital*) Annamalai University, Annamalai Nagar.

Antimicrobial activity:

Petri dishes with nutrient agar and Potato Dextrose Agar (PDA) were inoculated with five different species of bacteria and fungus. *S. javus* spine extract were sterilized by passing each through a 0.22 m Millipore GV filter (Millipore, U.S.A). Round paper discs with a radius of 0.8 cm were dipped into each extract of different concentration of 5mg/ml and 10mg/ml and placed in the center on inoculated petridishes.

Figure.1. Description of the study area of *Signaus javus*



The bacterial and fungal colonies were allowed to grow overnight at 37ºC and 20ºC respectively, and then the inhibition zone around the disc was measured.

Hemolytic assay

The hemolytic activities of crude extracts of *S. javus* were assayed on chick, goat, sheep and human erythrocytes followed by the method (Paniprasad and Venkateshwaran, 1997).

High Performance Liquid Chromatography

The crude spine venom was fractioned by analytical HPLC using Shimadzu C - 18 column with two solvent systems: **a.** 0.1% TFA solution **b.** 0.1% TFA in 90% Acetonitrile. The column was eluted at a flow rate of 1ml/min with 10 - 90% gradient solution B over 40min of total volume of 20µl. The RP - HPLC column elutes was monitored by their absorbance at 215nm and 280nm.The amino acids present in the sample were determined by comparing the Rf value of band formation sample with that of 21 standard amino acids.

Fourier Transform-Infra Red spectrum analysis

FT-IR spectroscopy of solid samples of *S. javus* venom relied on a Bio - Rad FT - IR - 40 model, USA. The rabbit fish venom (10mg) was mixed with 100mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc (10mm diameter) for reading the spectrum further.

Anticancer assay

Cell line and culture

Vero and Hep$_2$ cell lines were obtained from Tamil Nadu Veterinary College, Chennai. The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 µg/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

MTT assay- Cytotoxicity activity

The Cytotoxicity of samples on Vero and Hep_2 were determined by the MTT assay (Mossman, 1983). Cells (1×10^5/well) were plated in 1ml of medium/well in 24 - well plates (Costar Corning, Rochester, NY). After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate - buffered saline (pH 7.4), 200µl/well (5mg/ml) of 0.5% 3- (4, 5- dimethyl - 2 - thiazolyl) - 2, 5-diphenyl - tetrazolium bromide cells (MTT) solution was added. After 4h incubation, 0.04M HCl/isopropanol were added. Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a UV - Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of Vero and Hep_2 were expressed as the % cell viability, using the following formula:

$$\% \text{ cell viability} = \text{A570 of treated cells} / \text{A570 of control cells} \times 100\%.$$

Statistical Analysis

Tests were carried out in triplicates. The mean values were calculated from the triplicate values. Values are expressed as the mean ± SD and differences between groups were considered to be significant if $p<0.05$.

Results

Preparation of Crude Extracts

Crude extracts yield a total amount of 2.45g of crude extract from 50g of spine. The protein content of *S. javus* crude extract was found to be 2.4 mg/ml its shown in figure 3.

Antimicrobial Activity

The effect of the spine venom of rabbit fish on pathogenic bacteria and fungi reveled that, there samples were shows significant activity. The obtained results were represented in figure 4.

Hemolytic Assay

The results of the hemolytic assay on chick, goat, sheep and human blood sample erythrocyte were done using PBS crude extracts. The results were shown in figure 5. The result reported that spine extracts showed a very strong hemolytic activity in human 16 HU and sheep blood 14 HU, moderate activity in chick blood 10 HU and lowest activity in goat blood 8 HU respectively.

FTIR

FTIR is the most useful for identifying chemicals that are either organic or in organic. The crude extract showed varied range of peaks which consists of nitro compounds, sulphates, phosphates and methylene groups. Spine venom consists of heterocyclic amine NH stretch, primary amine CN Stretch at 3423 cm^{-1}, 1047cm^{-1}, in addition it contains asymmetrical / symmetrical C-H stretch of methyl and methylene, isothiocanate, aliphatic nitro compounds shown in figure 6.

HPLC

The amount of amino acids present in crude extract was varied for 20 amino acids (Table 3). Amino acid content of crude extracts was analyzed by using HPLC by comparing the Rf values of 20 amino acids. Particularly, the spine amino acid composition were rich in Serine, Threonine, Glutamic acid, Tyrosine, Histidine, Phenylanine and Glycine but low in basic amino acids shown in figure 7 and 8.

Cell line and culture

The cytotoxicity of spine venom were studied on Hep_2 cell and Vero cell by using MTT assay at various concentrations of sample .The Viability of Hep_2 Cell and Vero cell were adversely affected upon adding crude extracts . The relative cytoxicity on both cells were illustrated in figure (9 and10). The toxicity symptoms shown by the cells were lysis and detachment from substratum. Hep_2 cell and Vero cells showed venom concentration - dependent cell death and their $\mathbf{IC_{50}}$ values were calculated. The $\mathbf{IC_{50}}$ values of crude spine venom on Hep_2 Cells was found to be 51 µg/ml and on Vero cells 50 µg/ml a respectively. The cells were carefully observed using inverted Microscope and photographed was shown in figure (11 and 12).

Figure.2. Shows the fin locator of venomous spines *signaus javus*

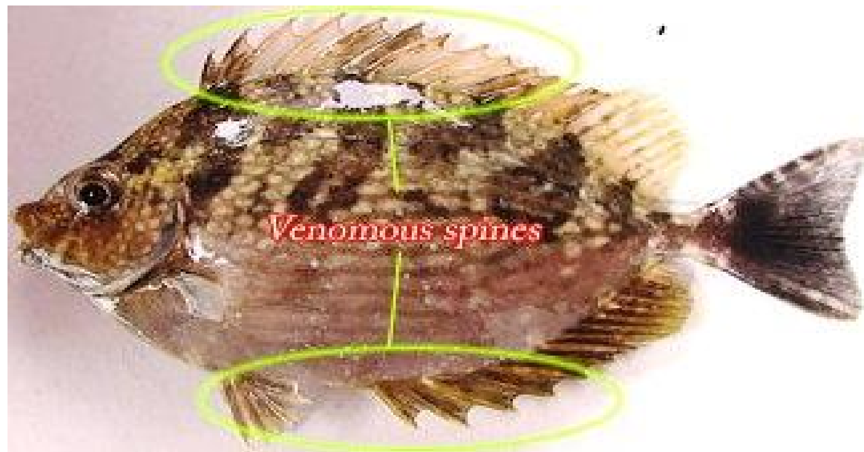


Figure 3: Shows crude extracts and protein content of *signaus javus*

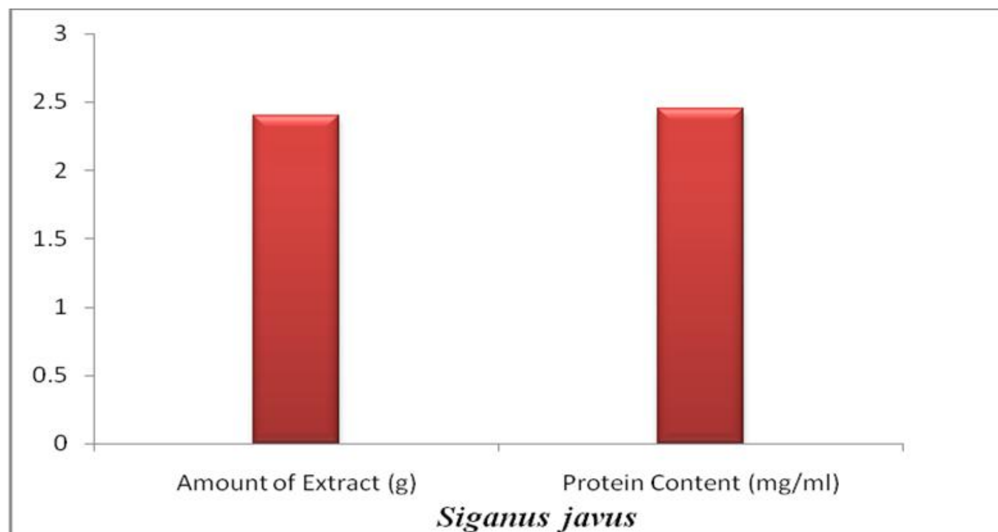


Figure 4. Shows antimicrobial activity of *Signaus javus*

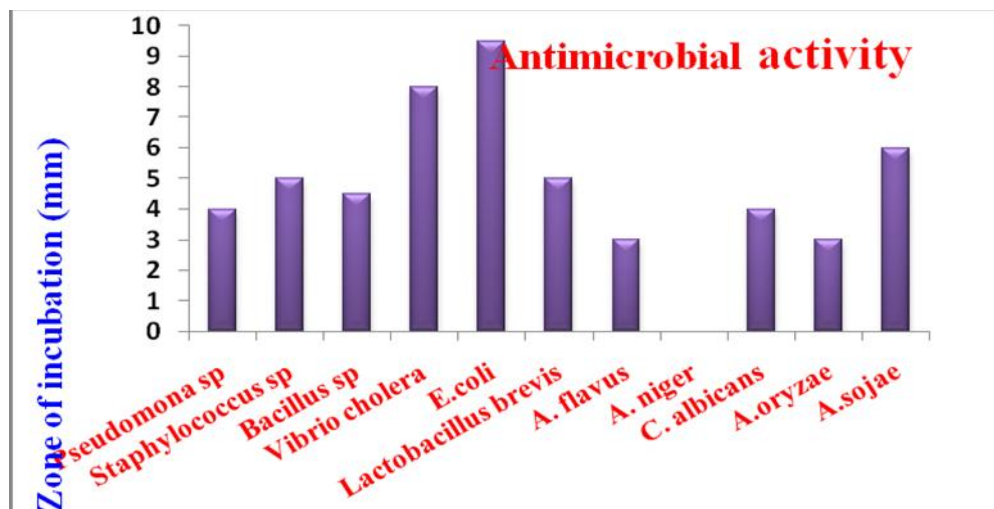


Figure. 5: Shows the hemolytic activity of *Signaus javus*

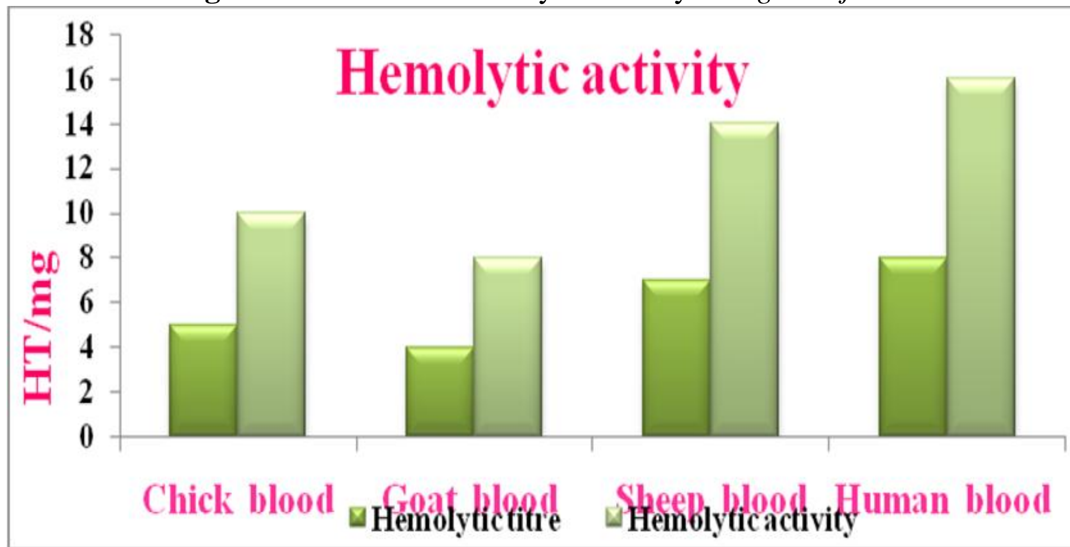


Figure 6: Shows the functional groups of *Signaus javus*

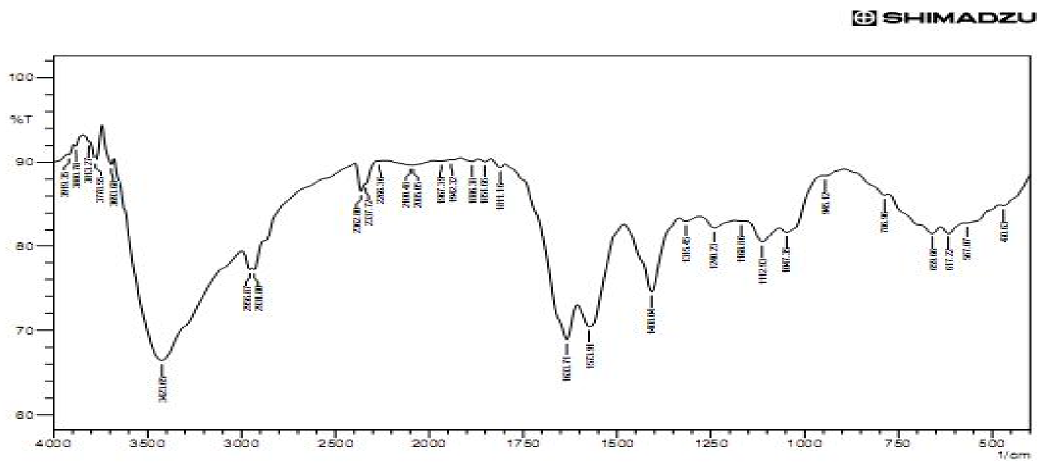


Figure 7: Standard graph of amino acid profile

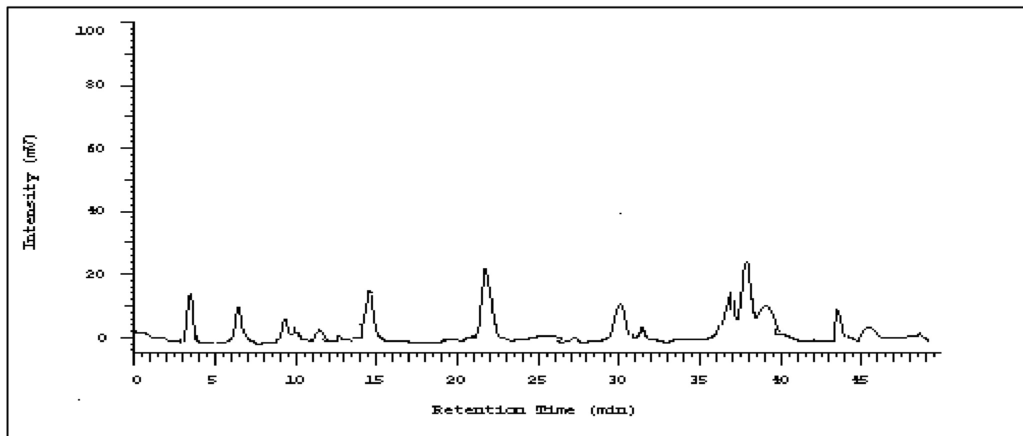


Figure 8: Show the amino acid profile of *Signaus javus*

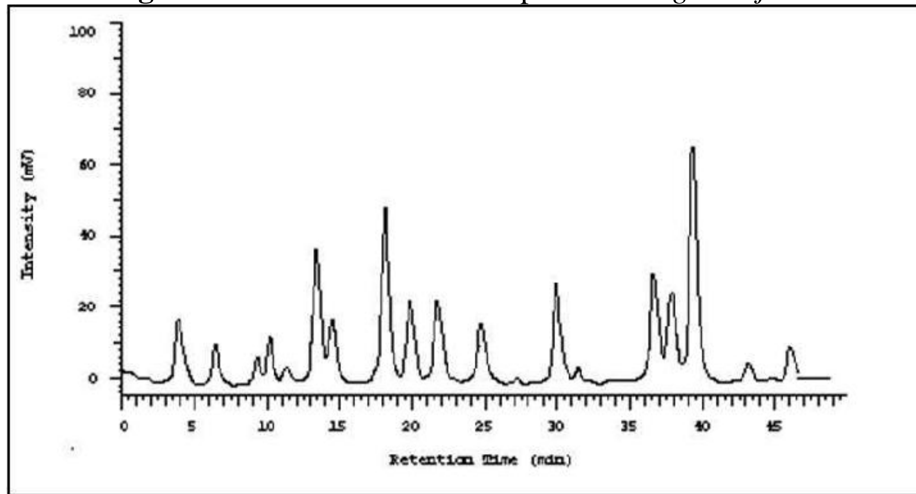


Figure : 9 Cytotoxicity effect of *Signaus javus* on Vero Cell line

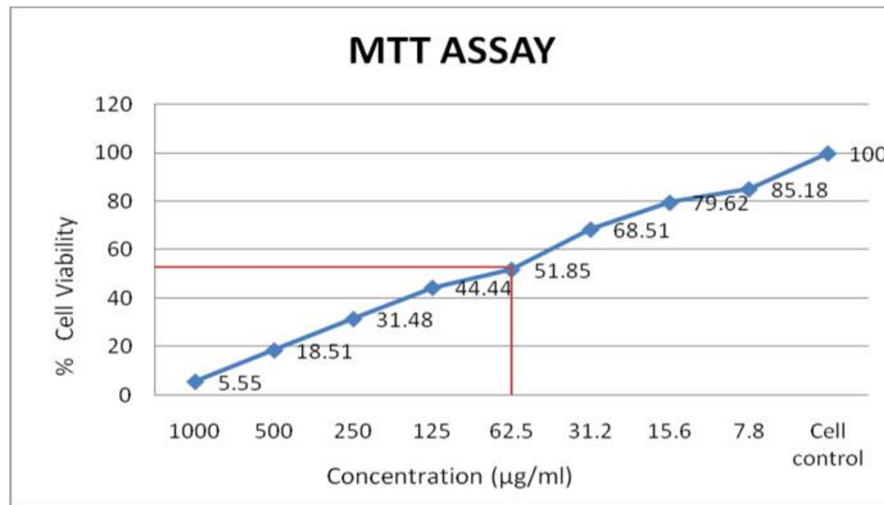


Figure : 10 Cytotoxicity effect of *signaus javus* on Hep2 Cell line

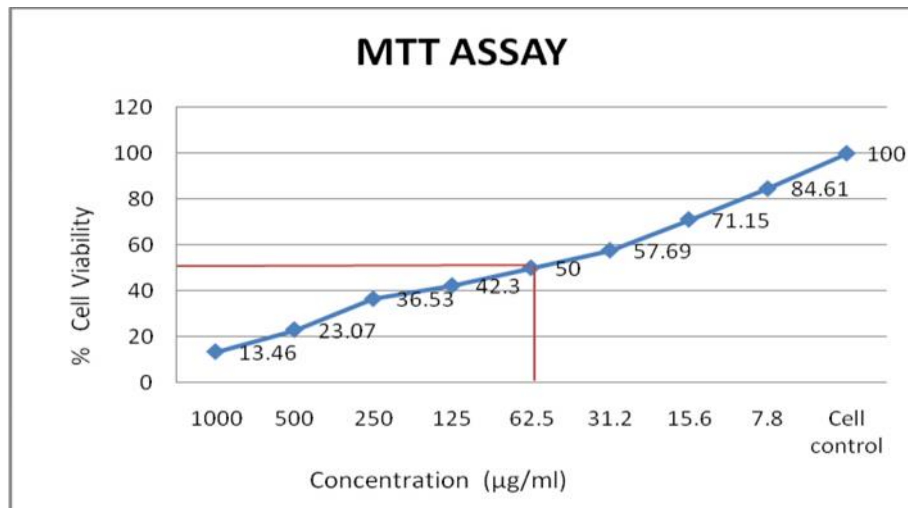


Figure: 11 Cytotoxicity effects of *Signaus javus* on Vero Cell line
Normal Vero Cell Line

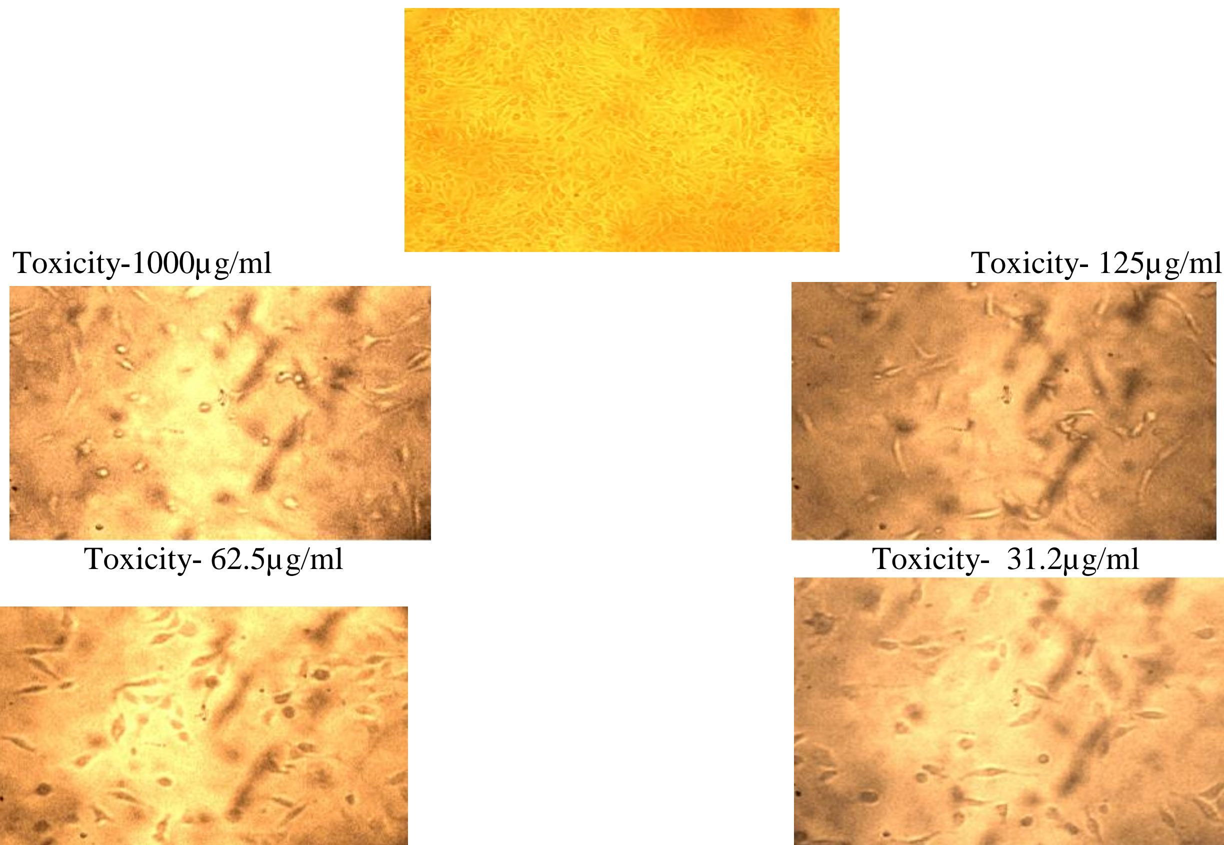
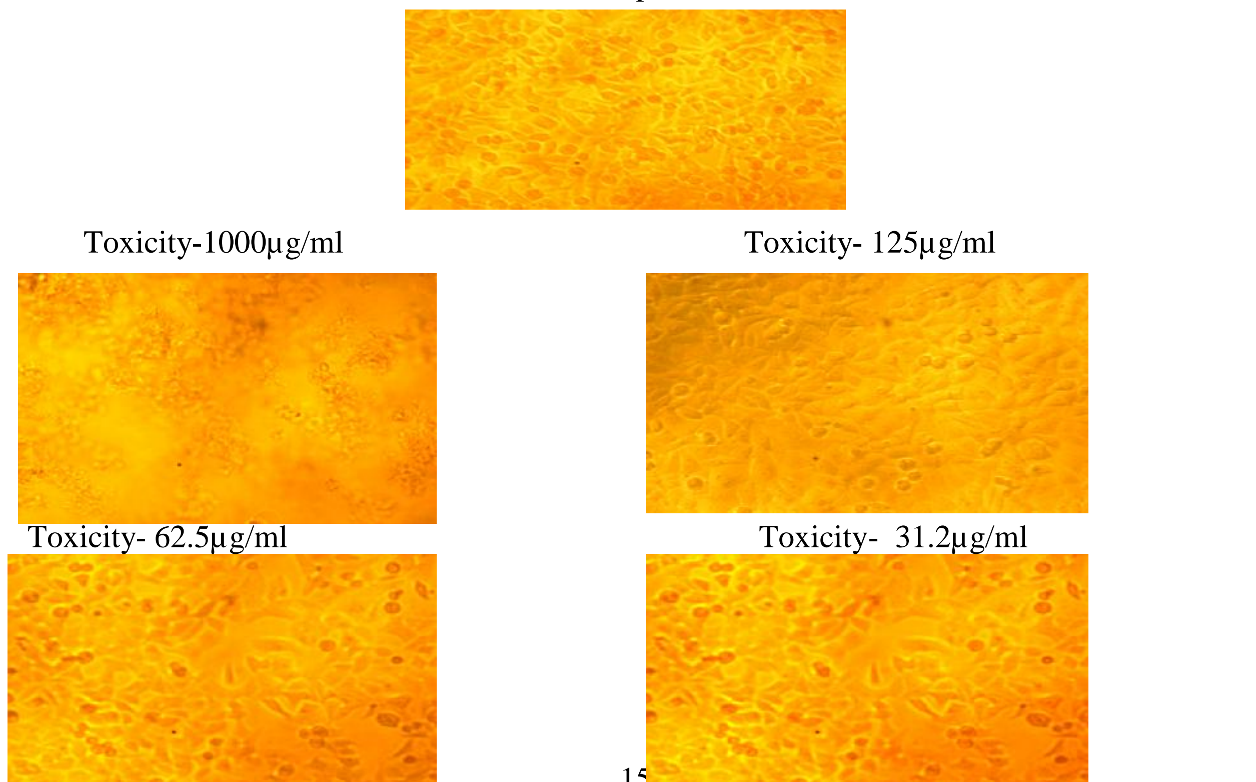


Figure: 12 Anticancer effect of *signaus javus* on Hep2 Cell line
Normal Hep 2 Cell line



DISCUSSION

In general, epidermal toxins and venoms of poisonous fishes are produced by the protein elaborating cell, epidermal secretions contain a mixture of highly active biochemical and pharmacological components that are different from typical fish mucus which is composed predominately of glycoprotein (Al Hassan *et al*., 1982). Thus knowing the importance of toxins and venom the present study was carried to investigated an *in vitro* antimicrobial activity and bioactivities of protein isolated from rabbit fish *S. javus.*

In crude extraction the amount of protein obtained from 50g of fish spine was 2.45µg/ml respectively. Similarly, the biochemical composition of the crude mucus of the catfish *A. maculates* shows protein as the major component. The concentration of protein in the insoluble and soluble fractions was found to be 12.6 and 9.34 mg/g respectively (Manivasagan *et al*., 2009). The mucus of catfish *A. thalassinus* is a mixture containing over 60m proteins and a variety of other components, including sugars, lipid, nucleotides and nucleosides (Al Hassan and Ali, 1985).

In the present study, the crude extract venom showed remarkable antimicrobial against bacteria and fungi. The gels of Atlantic salmon showed broad antibacterial activity suggested that it may play an important role in non specific immunity in catfish (Randall, 1995). The lytic activities of gel proteins may have a role in defense against microorganisms (Al Hassan and Ali, 1985).

Amino acids are building blocks of proteins and serve as body builders. They are ultilized to form various cell structures, of which they are key components and they serve as source of energy (Babsky *et al*., 1998). Thus the amino acid composition of rabbit fish *S. javus* spine venom consists of different amounts of essential amino acids. The skin toxins consists of all amino acids among these lysine, lecine, and aspartic acid are the major amino acids and proline tryptophan was found as lower amino acids from this above result *S. javus* spine amino acid composition were rich in Serine, Threonine, Glutamic acid, Tyrosine, Histidine, Phenylanine and Glycine but low in basic amino acids. This suggests that the enzymic activities of spine venom differ based on amino acid composition. Previously Al Hassan and Ali (1985) reported that amino acid analyses of both soluble and insoluble gel protein of skin secretion of Arabian gulf catfish *A. thallasinus* yielded similar results. Further the toxin from the red sea flat fish is a hydrophobic acidic protein (paradoxin) with a molecular weight of 17,000 Da was composed of 162 amino acids (Paniprasad and Venkateshwaran, 1997; Primor *et al*., 1980).

The crude rabbit fish venom showed a varied range of peaks which consists of nitrocomponds, sulphates, phosphates and methylene. As such spine venom consists of heterocyclic amine NH stretch at 3435cm, 1048cm. In addition it contain asymmetrical /symmetrical C- H stretch of methyl and methylene, methoxy, methylether, isothiocyanate of S -S stretching, aldehyde, alkenyl = C stretch, asymmetrical /symmetrical aliphatic nitro compounds of X02 stretch sulfonates, dialkyl/acyl sulfones and aliphatic nitro compounds of C - I stretch. More recently the FTIR analysis of mucus of two marine fishes *Cynoglossus Arel* and *Arius Caelatus* shows distinct spectral profile which confirms the presence of primary amine - group, aromatic - compound, halide- group, aliphatic alkyl - group and polysaccharides (carbohydrates) (Primor Parness and Zlotkin, 1978).

The crude venom of rabbit fish *S. javus* posses hemolytic activity on chicken, sheep, goat and human blood. Spine extracts showed a very strong hemolytic activity in human 16 HU and sheep blood 14 HU, moderate activity in chick blood 10 HU and lowest activity in goat blood 8 HU respectively. Earlier the specific activity of skin secretion Arabian gulf catfish *A. thallasinus* purified peak 5 fraction on sheep red blood cells was nearly 14 hemolytic units (HU)mg^{-1} protein (Al Hassan and Ali, 1985). Almost all piscine venoms posses hemolytic activity (Khoo *et al*., 1992; Kreger and Molgo, 1993; Chhatwal and Drcyer, 1992; Garnier and Goudey, 1995). The mechanism of hemolytic activity of stone fish venom have been shown to

produce hemolysis by forming hydrophilic pores in cell membrane ,which results in cell lysis (Chen *et al*., 1997). A new vasoactive cytolytic toxin (SP-CTX) exhibited a potent direct hemolytic activity (EC 5056 mg/ml = 0.46nm) upon washed rabbit erythrocytes (Babsky *et al*., 1998), which is comparable to the hemolysis induced by SNTX (EC 50 60 mg/ml) (khoo *et al*., 1992) and neo VTX (EC50 83 mg/ml) (Stempein *et al*., 1970).

MTT Assay originally developed for cell viability testing is now widely used in cancer cell cytotoxicity testing (Mossman, 1983). In the cytotoxicity studies, the IC_{50} vale of spine of rabbit fish on Hep_2cells was found to be 51 µg/ml. and same on Vero cells as to be 50 µg/ml a respectively. The control group doesn't show any considerable cell death. There results infer that Hep_2cells appeared to be much susceptible to crude samples of spine venom when comparing with Vero cells. In the Vero cells at the susceptible concentration of cancer cells crude samples shows about 70% of viability, the higher concentration of spine venom induced more serious morphological alteration in both cell lines. This cell death may be due to apoptosis or necrosis. Cytotoxity has also been recently reported for fish venoms of *Thalassophryne nattereri* on endothelial cell lines of capillary origin (Lopes Ferreira *et al*., 2002) and *Gadopsis marmoratus, Pterois volitans and Synanceia trachynis* on cultured murine cortical cells (Church *et al*., 2003). Earlier, the Hela cells incubated with *S. argus* venom of different concentrations for nearly 4 hour found the cells distended (Gisha sivan., 2007). Recently the cells were distended after exposure to fish venoms of the family scorpaenidae, supporting the hypothesis that depolarization of the cell and ionic imbalance lead to an influx of fluid into cytosol resulting in swelling of cells and eventually cell lysis (Church *et al*., 2003).

Conclusion

Thus the present study gives us baseline information about characterization of spine venom of the *S. javus* rabbit fish The bioactivity of the sample differs because of extreme liability of fish venom toxins even their biological activity is lost during storage. There results also suggest that the cause serve envenomation upon introduced into an open skin not only responsible by spine venom but also accompanied by mucus secretion that enters wound at the time sting increase virulence effect. The present investigation conclude that bioactive compounds are present in crude extract were responsible for biological activity such as antimicrobial, hemolytic, antioxidant, anticancer activity. Thus these biological properties of rabbit fish *S. javus* spine venom may pave a way for the isolation of specific protein or novel compounds and or development of new therapeutic strategies complementary to conventional therapy. Further detailed studies could be made on *S. javus,* which may lead to the discovery of new potent drugs in future.

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