Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(10):254-259

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Antimicrobial activity of *Scorpaenopsis venosa t*oxic extracts and its structural elucidation

R. Sasikala and Vinoth S. Ravindran*

Suganthi Devadason Marine Research Institute, 44, Beach Road, Tuticorin

---

ABSTRACT

The Scorpion fish, Scorpaenopsis venosa collected from Kanyakumari coast, Tamil Nadu, West Coast of India was identified based on the morphological characters. The antibacterial assay with crude extracts and purified fractions of venom gland and gonad extract was carried out with six bacterial strains viz., Pseudomonas sp. Staphylococcus aureus, Vibrio harveyi, V. parahaemolyticus, Proteaus sp., and Escherichia coli. Antagonistic activity was noted against most of the bacterial species. The crude and fractions of gland and gonad, was potent against V. harveyi. The antifungal assay was carried out with three fungal strains viz., Rhizoctonia solani, Pythium proliferum and Aspergillus niger. Venom gland fractions inhibited the growth of Rhizoctonia solani. Other two fungi were resistant to venom gland and gonads crude extracts and fractions. The FTIR analysis of both the samples showed various peak values aligned as halide, amine, ether and alcohol.

Keywords: Anti-fungal, Antibacterial, Fourier Transform Infra-Red, Gonads, Venom glands

---

INTRODUCTION

Fishes are one of the diverse sources 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.

There are more than 200 fish species reported to produce venoms, but less than 20 have been studied in some detail. Most of these venoms appear to comprise proteins and peptides as well as other pharmacologically active substances. The significance of fish venoms both for research and medical therapeutic purposes and as chemical defences remains poorly appreciated. The number of compounds isolated from various marine organisms has soared from 10,000 in 2001 to 14,000 in 2007. The Raggy scorpion fish *Scorpaenopsis venosa* is considered to be one of the dangerous venomous fishes in the world and certainly the most venomous in the *Scorpaenidae* family. Therefore, the present investigation was undertaken to elucidate the bioactive properties of the venom of *S. venosaviz.*, antibacterial and antifungal. The structural elucidation of the most active fractions was done using Fourier Transform Infrared Spectroscopy (FTIR).

EXPERIMENTAL SECTION

Collection of *Scorpaenopsis venosa*

Scorpaenopsis venosa were collected from Kanyakumari coast, Tamil Nadu, West Coast of India with buckets and aerators supplied to the fishermen brought live to the laboratory and identified [1].

Extraction of venom from venom gland
Fish were chilled at 20˚C for 10-20 min and then decapitated; the dorsal spines were cut approximately 3-5 mm from their base. The spines were homogenized in 0.9% NaCl. The supernatant was centrifuged for 15 min at 6000xg to remove insoluble materials. The resulting supernatant was designated as *Scorpaenopsis venosa* extract. All steps were performed in a cold room at 4-10˚C. Freshly extracted soluble fraction was used for all experiments [2].

Extraction of gonad toxins
The gonads of *S. venosa* were dissected and the extract was prepared by squeezing the sand-free specimens in triple distilled water. The resultant solution was filtered and it was again squeezed with Phosphate Buffer Saline (PBS). The supernatant so obtained was lyophilized (Labcono Freeze Dry System) and stored at -20˚C until further use.

Partial purification of the crude extract using column
Partial purification of the crude extract of venom gland and gonad was carried out using DEAE Cellulose Anion Exchange chromatography [3]. Four fractions were collected in a step-wise gradient with NaCl and stored at -20˚C for further use.

Protein estimation
Protein estimation [4] was done using Bovine Serum Albumin as standard. The absorbance was read spectrophotometrically at 280nm.

Artemia Bioassay
The Artemia bioassay was conducted [5], [6]. The dried [7] *Artemia* cysts were cultured in culture bottles and the cysts were incubated (1g/L) at 28-30˚C with strong aeration, under permanent light conditions. Approximately 24 hrs after hatching the phototrophic nauplii were collected using a sterile pipette from the lighted side and concentrated in a small vial. The nauplii were divided into many groups of 10 individuals each in 10 ml sterile seawater. The extracts at varying concentrations (5, 7.5, 10 and 15 µg/10ml) were added into vial with *Artemia*nauplii. Also *Artemia* larvae were exposed to the extracts in another set of experiment. Controls were maintained separately. Toxicity was determined after 24 hours of exposure. The surviving *Artemia* were counted and % death was calculated. Larvae were considered as dead if they did not exhibit any internal or external movement during observation. Experiment was carried out in triplicate for each concentration and average was taken and LC_{50} was determined.

Antibacterial activity
Antibacterial activity was assessed by agar well diffusion method [8]. The potency of the venom gland and gonad toxins against bacteria was checked on *Pseudomonas sp., Staphylococcus aureus, Vibrioharveyi, V. parahaemolyticus, Proteaus sp.,* and *Escherichia coli.* Lawncultures of these strains were prepared by pouring 2 ml of each culture in 16-18 hours Nutrient Broth over Muller Hinton Agar Media plates separately and they were swabbed. The plates were air dried by keeping in an incubator at 30˚C for 15 minutes. 3mm diameter wells were punched in the plates using a sterile gel puncture. Subsequently, 50 µl of purified extracts were directly added on to the paper disc and incubated for 18-20 hours. Antimicrobial activity was assessed by observing the suppression of bacterial growth on application of the sample to the top agar surface. Then the inhibition zones (in diameter) were noted.

Minimum inhibitory concentration (MIC) determination
Serial tube dilution technique was used to determine MIC of the compound against these bacteria. 100µl of DMSO was added to the 96 well microtitre plates. Then added 100µl of purified venom gland and gonad fractions to the wells. Serially diluted the samples to each well and added 100µl of Muller Hinton broth into each well. Incubated it for 24 hours. After incubation 20µl of 0.5% Tetrasolium salt was added. After five minutes the colour formation was noted. Pink colour formation indicates growth of the bacterial colonies. No colour change indicates the absence of bacterial growth. Distilled water with few drops of Tween 80 and Kanamycin were used as negative and positive controls, respectively.

Antifungal activity
5g of Sabouraud Dextrose Agar Media was dissolved in 100 ml of distilled water. The pH was adjusted to 8.0-8.4 with 5 M NaOH and boiled for 10 minutes. It was filtered and sterilized at 115˚C for 30 minutes. The pH was then adjusted to 5.6.

A suspension of the organisms *viz., Rhizoctoniasolani, Pythiumproliferum*and *Aspergillusniger* were added to the Medium at 45˚C. The mixture was transferred to sterile petriplates and allowed to solidify. Sterile 5 mm diameter

discs were loaded with 1ml of crude and fractions of venom gland and gonad separately. The discs were placed in the agar plates. Then the plates were incubated for 48 h at 37˚C and observed for antifungal activity. Clear zones were measured and recorded.

FTIR analysis

The DEAE cellulose column most active fractions of both organs (venom gland and gonad) were analyzed qualitatively for the active compounds by Fourier Transform Infra-Red (FTIR) spectrometry [9].

RESULT AND DISCUSSION

Extraction of crude venom from venom gland and gonads

S. venosa organs (venom gland and gonads) were dissected and extracted. Lyophilized extracts yielded a total amount of 5.4 g of crude venom from 500 g of crude venom gland extract. 30 g of gonad extract yielded 3.09 g of lyophilized protein. A two-step procedure on Sephacryl S-200 high-resolution gel-permeation and DEAE Bio-Gel anion-exchange chromatography of Stone fish *S. horrida* venom has evolved similar results [10]. The present lyophilized samples were also purified using DEAE-cellulose anion exchange chromatography.The present research[11] highlights the nutritive value of the meat of *P.trapezium* such as its protein, low lipid, vitamins, minerals and trace metal contents and Polyunsaturated Fatty Acids (PUFAs) like, Linoleic, Linolenic, Eicosapentaenoic acid (EPA) Arachidonic and Eicosatrienoic acids. The meat also has good antioxidant activity, hence justifying the need to popularize this meat as important seafood.

Protein estimation

The protein content in crude venom gland and gonad extracts were 4.35 and 3.90 $mg.mL^{-1}$ respectively. The protein in purified fractions ranged between 0.04 and 0.076 $mg.mL^{-1}$ for venom gland extract and 0.06 and 0.9 $mg.mL^{-1}$ for gonad extracts. The protein concentration is drastically higher in the case of liver extract than the skin extract of puffer fish *Takifugurubripes,* whereby the liver extract shows 0.98 $mg.mL^{-1}$ of protein concentration and the skin shows 0.45 $mg.mL^{-1}$[12]. The protein in venom gland extract of *S.horrida* has been earlier reported to be 1 $mg.mL^{-1}$ [10].

Artemia Bioassay

The artemia toxicity experiment shows that a toxin extracted from S.venosa is toxic to artemia larvae at all the tested concentrations; hence the LC_{50} has been arrived. These results are similar and comparable to an earlier study [13], which reported the effect of TTX on the larval development of penaeid shrimp (*Metapenaeusensis*). It is reported that TTX inhibited the heartbeat of later juvenile instars. The locomotoractivity[14] of the animal was reduced by the extracts of the *D. margariticola* and *T. tentorium*. But the activity was found to be dose dependent, the higher the dose the lower the activity. In control, the activity was found to be very negligible. The standard Chlorpromazine treated animal exhibited a nearly 67.86% reduction in locomotor activity.

The LC_{50} values of toxin extracted from fish parts *viz.,* venom gland and gonads for both crude and pure extract are shown in Table 1. The venom gland and gonad extracts showed high toxicity in the artemia assay.

Table. 1- Artemia toxicity for crude and pure extracts

***Scorpaenopsis venosa* extracts**	**Fractions**	**Protein content (mg/ml)**	**Artemia toxicity LC_{50} (µg/ml)**	
			Crude	**Fractions**
venom gland	FR1	0.076	1.5 ± 0.15	1.5 ± 0.12
	FR2	0.053	1.25 ± 0.12	1.25 ± 0.9
	FR3	0.039	1.65 ± 0.2	1.65 ± 0.21
	FR4	0.04	1.5 ± 0.18	1.5 ± 0.15
Gonad	FR1	0.90	1.85 ± 0.15	1.65 ± 0.12
	FR2	0.061	1.5 ± 0.18	1.5 ± 0.17
	FR3	0.076	1.25 ± 0.8	1.5 ± 0.13

Antibacterial assay

The crude and purified fractions of venom gland and gonad extracts of *S. venosa* produced zone of inhibition against pathogens *viz., Pseudomonas sp., Staphylococcus aureus, Vibrio harveyi, V. parahaemolyticus, Proteaus sp., and Escherichia coli* (Table 2). The purified extracts of spine formed maximum inhibition (15.5 mm) against *Vibrio harveyi* in fraction II and minimum inhibition (6.5 mm) against *Staphylococcus aureus*in fraction II. Fraction III of gonad produced maximum inhibition (18 mm) against *Vibrio harveyi* and Fraction III produced minimum (6.0 mm) against *Staphylococcus aureus.* The antibacterial role of fish mucous against marine microbes is well established.

The epithelial tissues produce antimicrobial moleculeswhich serve as the first line of defence against microbial invasion in a variety of vertebrates including humans. The skin homogenate of the brown-spotted grouper, *Epinephelusfario* is reported to contain an antimicrobial compound [15]. Toxins derived from jellyfishes have been exploited [16] as a model for the development of new drug promising applications to treat neurodegenerative diseases. It has been reported that the crude venom of *Pelagianoctiluca* is found to be a useful tool for probing pharmacological activity. The purification and the determination of chemical structures of compounds of active fractions of the venom are under investigation.

Minimum inhibitory concentration

The MIC value against bacteria ranged from 1,000 to 60µg/ml, respectively (Table 3). MIC of puffer fish *A. reticularis* liver extract against *S. aureus* was recorded as 150 µL and MIC of skin extract against *V. cholera* was found to be 260 µL. It is also reported that the cell free extract of associated bacteria exerts antimicrobial sensitivity against microbial pathogens. This is also supported by the earlier studies that, the amoebocytes from the sea anemone *Actinia equina* showed considerable inhibitory activity against Gram-negative bacteria [17].

Table. 2-Antibacterial activity of spine and gonad fractions against human bacterial pathogens

Pathogens	Zone of inhibition (mm)						
	venom gland				Gonad		
	FR1	FR2	FR3	FR4	FR1	FR2	FR3
E. coli	12.677±2.08	7.53±0.55	-	-	-	-	-
Pseudomonas sp.	-	-	-	-	15.5±1.0	15.5±0.56	18 ±2.0
Proteaus sp.	-	14.5±1.5	-	10.5±1.0	12±1.5	9±0.5	-
Staphylococcus aureus	-	6.5±0.5	-	-	7±0.5	16.5±2.5	6.0±0.5
Vibrio harveyi	11.5±1.0	15.5±1.5	12.5±1.5	13.0±2.0	15.5±1.5	16.5±1.5	18±2.5
Vibrio parahaemolyticus	-	-	-	8.5±1.5	9.5±1.0	-	-

FR-Fraction

Table. 3- Minimum Inhibitory Concentration of Active fractions

S.No.	Pathogens	Minimum Inhibitory Concentration (µg)						
		Extracts						
		venom gland				Gonad		
		FR-1	FR-2	FR-3	FR-4	FR-1	FR-2	FR-3
1	*Escherichia coli*	125	500	500	250	125	250	500
2	*Pseudomonas sp.*	250	250	500	500	500	250	500
3	*Proteaus sp.*	60	60	125	125	250	250	250
4	*Staphylococusaureus*	60	125	125	125	125	250	500
5	*Vibrio harveyi*	125	60	125	125	60	60	1000
6	*Vibrio parahaemolyticus*	60	125	125	60	250	250	500

FR-Fraction

Antifungal activity

The results of the antifungal activity of the crude and purified spine and gonad extracts of *S. venosa* were recorded. It was observed from the present study that only fraction II of spine extract inhibited the growth of *Rhizoctonia solani*. Antimicrobial assay [18] in puffer fish *Arothron immaculatus* was screened with ten different fungal strains *viz., Aspergillus niger, Candida albicans, Aspergillus flavus, Mucoramphibiorum, Alternaria alternata, Pencillium chrysogenum, Rhizopusa zygosporus, Trichophytonrubrum, Trichopyton mentagrophytes, Epidermophyton floccosum*. But there is no report of antifungal activity.

FTIR Analysis

The Fourier Transform Infra-Red Spectroscopy (FTIR) analysis peak values are shown in Table.4 and Figure 1&2. The analysis of spectrum of fraction 2 of venom gland showing maximum antifungal activity revealed the following groups. Signals in 1639.38 cm^{-1} region is indicative of the group RON=O. The peak at 2104.19cm^{-1} is $R_2C=N=N$. Signals in the 3427.27 region are characteristics of $RCONH_2$, RCONHR′ groups. These are the functional group of amides.

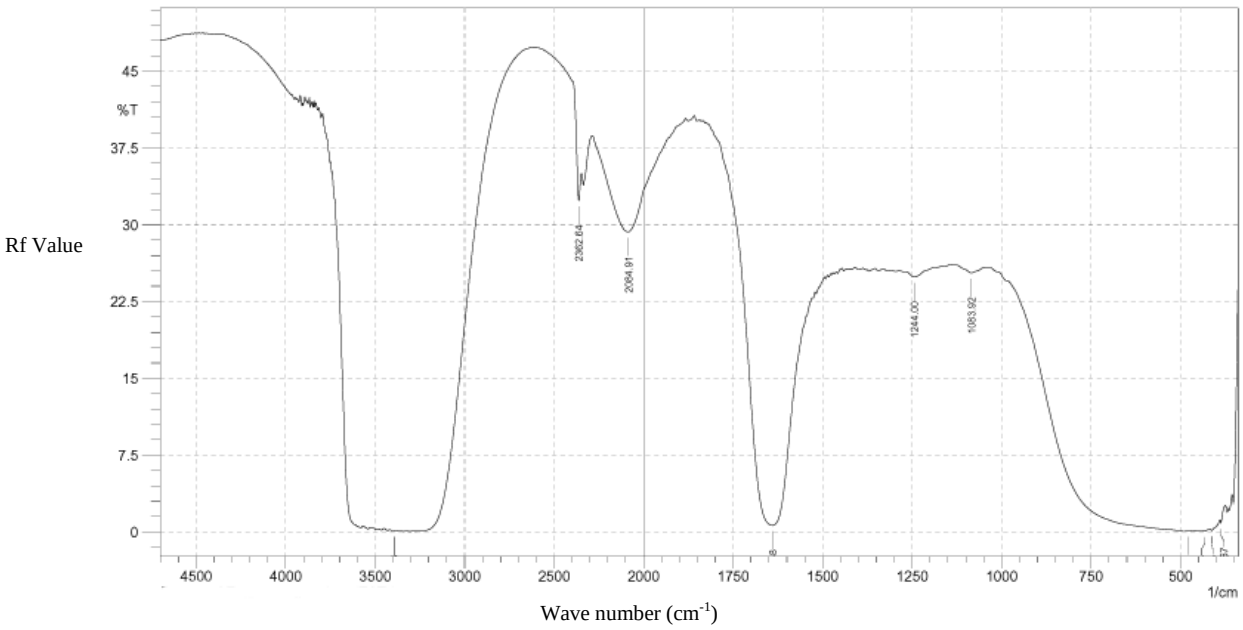

Fig 1- Absorbance, expressed as wavelength (cm^{-1}), obtained by Infrared spectroscopy of most active fraction II of venom gland in antifungal assay

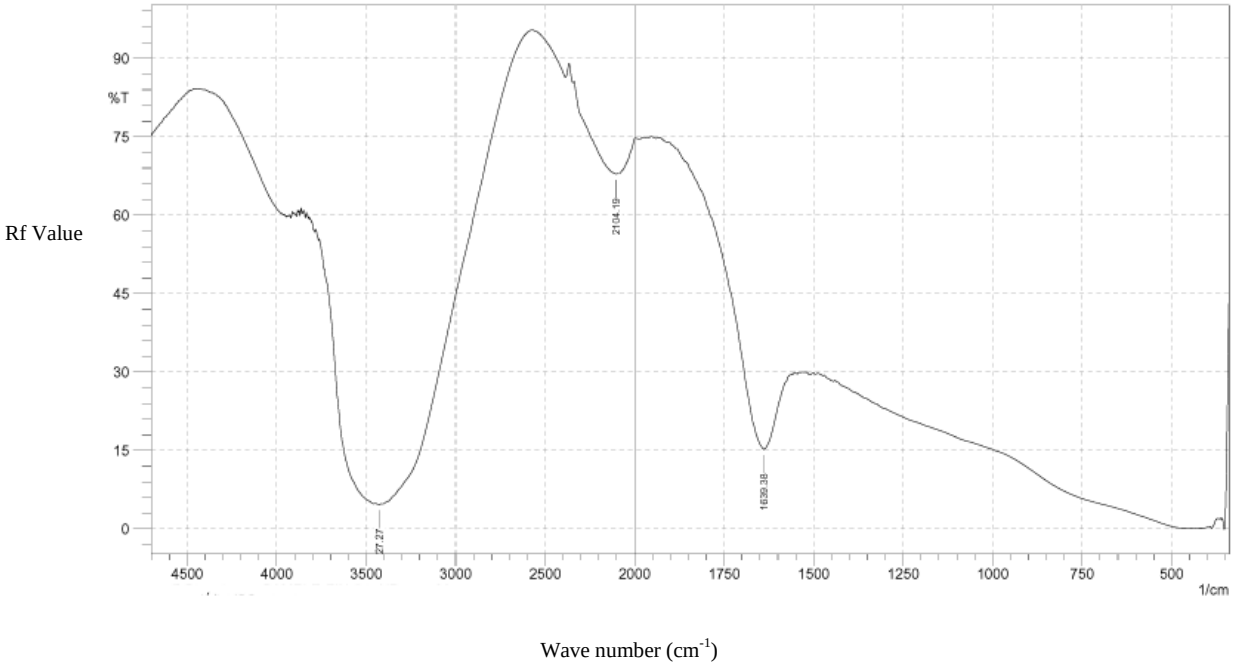

Fig 2- Absorbance, expressed as wavelength (cm^{-1}), obtained by Infrared spectroscopy of most active fraction III of gonad in antibacterial assay

The spectral analysis of fraction III of gonad showing maximum antibacterial activity revealed the following peaks and corresponding groups. They are the peak at 387.67 cm^{-1} characteristic of C-I, at 1641.31 cm^{-1} of C=N, a peak at 3431.13 cm^{-1}chaecteristic of N-H which is the functional group of amine. FTIR has been used extensively to study structure of proteins and lipids [18, 20]. FTIR has been used as a tool to study alteration of proteins, lipids, and nucleic acids due to arsenic intoxication in fishes. The molecular structure and molecular composition of the liver of the freshwater fish *Oreochrmis mossambicus* using FTIR spectroscopy [21] has been reported. The FT-IR spectrum of chitin was also confirming the presence of chitin in the shell of stomatopods. The results [22] of the present investigation pave way and provide concrete information for the utilization of chitin in the development of drugs, artificial bone and raw material for the food industries in the near future. In the present study [23] we infer that most of the amino acids present in methanol extracts are also present in methanol: chloroform extracts of different

molluscan species. As amino acids are building blocks of protein this will aid to the identification of proteins and the followed HPTLC method is very efficient for quantification of amino acids.

Table 4: FTIR Analysis of Active Fractions

Active Fraction	Peak values in cm^{-1}	Specific context
Antifungal Assay(Fraction II of venom gland)	1639.38	RO-N=O
	2104.19	R2C=N=N
	3427.27	RCONH2, RCONHR'
Antibacterial Assay(Fraction III of gonad)	387.67	C-I
	410.81	C-I
	1083.92	C-N
	1641.31	C=N
	2088.76	Nil
	3431.13	RNH2, R2NH

Acknowledgement
The authors thank Director, Suganthi Devadason Marine Research Institute (SDMRI), Tuticorin for facilities.

REFERENCES

[1]Cuvier, Le Règne; Animal, distribuéd'après son organisation, pour servir de base à l'histoirenaturelle des animaux et d'introduction à l'anatomiecompare.,**(1829),**2(1),166.
[2]Gisha Sivan, Venketesvaran K, Radhakrishnan C K;*Toxicon.*,**(2007),**50(1),563-571.
[3] Stempein M F, Ruggieri G D, Negrelli R F ,J T Cecil; *Marine tech Soc. Washington.*,**(1970),** 295.
[4]Bradford M M; *AnalytBiochem***, (1976)**,72(1), 248-254.
[5]Michael AS, Thompson CG, M Abramovitz;*Science*.,**(1956)**, 123(2),464–465.
[6]Vanhaocke P, Persoone G, Claus C,PSorgoloos;*Ecotoxicology Environmental Safety.*,**(1981),**5, 382–387.
[7]Carballo JL, Hernandez-IndaZL, Perez P,MD Garcia-Gravalos;*BMC Biotechnology*.**(2002)**, (2), 17–22.
[8]Perez, C, Paul,PBazerque; *Acta. Boil. Med. Exp.***(1990)**, 15,113-115.
[9]Kemp W;*Organic spectroscopyPalgrave, Published, New York.*, **(1991),**(3), 243-269.
[10]Poh C H, Yuen R, Khoo H E, Chung M, Gwee M ,PGopalakrishnakone;*Comp. Biochem. Physiol.,* (1991),99,793–798.
[11]Anand P T, Chellaram C, Kumaran S,C F Shanthini; *J. Chem. Pharm. Res.,* **(2010)**, 2(4),526-535.
[12]Matsumoto T, Tanuma D,K Tsutsumi;[*J*]. *Toxicon***.,(2010)**, **55** (2-3): 415-420.
[13]Mahon BR, Tanaka K, Doyle JE,KHChu;*Comp biochem A mol Physiol.*,**(2002**) 133(3):577-87.
[14]Chellaram, C, Anand P, Kumaran T, Kesavan, DS, G Priya; *J. Chem. Pharm. Res.*,**(2011)**, 3(1), 154-159.
[15]Zhang Y, Zou A, Manchu R, Zhou Y,S Wang; *Zoological Research.*,**(2008)**,29, 627−632.
[16] AyedY , DellaiA , MansourHB, BachaH, SAbid;*Annals of Clinical Microbiology and Antimicrobials*., **(2012),** 11:15.
[17]Hutton, DCM, V. J. Smith;*New*Jersy., pp. (1996),15-30.
[18]Kumaravel K, Ravichandran1S,Joseph F R S, Manikod D, Doimi M; *Chinese Journal of Natural Medicines.*,**(2011)**.
[19]Cakmak G, ToganI,F Severcan;*AqTox.*,**(2006)**,77, 53–63.
[20]Akkas S B, Severcan M, YilmazO,F Severcan;*FoodChem*, **(2007)**,105,1281–1288.
[21]Palaniappan PL RM,V Vijayasundaram;*Romanian J Biophys.*,**(2008)**, 18, 135–144.
[22]Thirunavukkarasu. N, Dhinamala K, Inbaraj R M; *J. Chem. Pharm. Res.*, **(2011)**, 3(1):353-359.
[23]Giji S, Abirami P , Arumugam M, T Balasubramaniam; *J. Chem. Pharm. Res.,* (**2011**), 3(5),93-100.